EVALUATION OF A SUBUNIT VACCINE TO INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Final Project Report

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INTRODUCTION

This project was funded because the incidence of infectious hematopoietic necrosis virus (IHNV) in fish at hatcheries along the Columbia River basin (CRb) had increased dramatically. The disease IHN threatened valuable hatchery stocks such as the Dworshak National Fish Hatchery steelhead and some of the prized chinook stocks in the CRb. Since there were no hatcheries for sockeye salmon in the CRb, the impact of this disease was not documented despite the fact that kokanee salmon were dying in large numbers each year in the CRb. A prototype IHNV vaccine had been developed at Oregon State University but many questions remained regarding the usefulness of such a vaccine. Obtaining answers to some of these questions formed the basis for the BPA contract, "Evaluation of a Subunit Vaccine to Infectious Hematopoietic Necrosis Virus."

There were six specific objectives and each of these objectives were realized.

1. Equip laboratory and hire personnel.
2. Evaluate different expression vectors for optimum vaccine production.
3. Evaluate immunization methods for IHNV vaccination.
4. Evaluate the immunogenicity of IHNV-specific proteins in salmon and trout.
5. Examine methods for vaccine preparation.
6. Prepare summary report.

In accomplishing the goals of the project, 15 scientific articles were published in refereed journals and five annual reports were also published by the Bonneville Power Administration. The scientific articles are part of this final report.

MANUSCRIPT # 1

Characterization of Infectious Hematopoietic Necrosis Virus mRNA Species Reveals a Nonvirion Rhabdovirus Protein

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Characterization of Infectious Hematopoietic Necrosis Virus mRNA Species Reveals a Nonvirion Rhabdovirus Protein†

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The genome RNA and six mRNA species of infectious hematopoietic necrosis virus were analyzed by denaturing gel electrophoresis. The following molecular weights were determined: genome RNA, 3.7×10^6; mRNA 1, 2.26×10^6; mRNA 2, 5.63×10^5; mRNA 3, 4.84×10^5; mRNA 4 (containing two different mRNA species), 3.00×10^5; and mRNA 5, 1.95×10^5. Densitometer analyses of gels were used to calculate the molar ratios of the intracellular mRNA species: mRNA 1, 0.02; mRNA 2, 0.49; mRNA 3, 1.0; mRNA 4, 2.52; and mRNA 5, 0.41. Hybrid selection studies determined the mRNA coding assignments as follows: mRNA 1 encodes the viral polymerase, L; mRNA 2 encodes the glycoprotein, G; mRNA 3 encodes the nucleocapsid protein, N; mRNA 4 is composed of two comigrating mRNA species which encode the matrix proteins, M1 and M2; and mRNA 5 encodes a previously unrecognized viral protein which is induced in infected cells but is not present in mature virions. This nonvirion protein has been designated the NV protein.

Infectious hematopoietic necrosis virus (IHNV) is a piscine rhabdovirus of economic importance as a pathogen of salmon and trout (2, 30). This virus is responsible for increasingly devastating losses of young fish at hatcheries in western North America (12). Survivors of a hatchery epizootic of IHNV appear to be virus free until they return from the ocean and begin to spawn (2). At that time, the virus appears in many tissues of the fish and is presumably transmitted vertically via the spawning fluids. There also is substantial evidence for horizontal waterborne transmission of IHNV (28, 30). The life cycle of IHNV in relation to its carrier host is not known (23, 28, 30).

IHNV is similar to the rhabdovirus prototypes, vesicular stomatitis virus (VSV), and rabies, in that it is a bullet-shaped, enveloped virus with glycoprotein surface projections and a genome of single-stranded, negative sense RNA (3, 13, 25). The protein structure of IHNV has been reported to resemble that of rabies virus and consists of a viral polymerase (L), a surface glycoprotein (G), two matrix proteins (M1 and M2), and a nucleocapsid protein (N) (13, 19, 23). Although the proteins of IHNV have been well characterized, the viral nucleic acid species have received little attention. A system for in vitro transcription of IHNV has been described previously (24), but individual transcripts were not resolved or identified. Therefore, to further our understanding of the molecular biology of IHNV both in the carrier state and during lytic infections, it was first necessary to characterize the viral genome and mRNA species.

We report here the isolation of genomic RNA from purified IHNV virions and six viral mRNA species from IHNV-infected salmon cells. These molecules were resolved by electrophoresis on both glyoxal and methyl mercury gels, and the molecular weights and molar ratios present in infected cells were determined. In addition, the mRNA species were characterized by hybrid selection and in vitro translation studies which resulted in the identification of the viral protein coded for by each message. Our results identify mRNA species for all the known viral proteins and, in addition, identify a message for a previously unrecognized viral protein which is present only in the infected cells.

MATERIALS AND METHODS

Cells and virus. The IHNV used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte Hatchery in central Oregon. The virus was propagated in a chinook salmon embryo cell line (CHSE-214) obtained from J. L. Fryer, Department of Microbiology, Oregon State University, Corvallis. Cell monolayers were infected with IHNV at a multiplicity of infection of 0.001 and incubated at 16°C for 7 days. When radioactive labeling of viral genome RNA was desired, [5,6-^{3}H]uridine (30 Ci/mmol; New England Nuclear Corp.) was added to 10 μCi/ml 24 h postinfection. The supernatant fluid was harvested and clarified of cellular debris by centrifugation at 2,400 × *g* (4,000 rpm) in an HS-4 rotor in a Sorvall RC5 centrifuge for 10 min at 4°C. Clarified supernatant fluids were stored at −20°C, or the virions were purified on discontinuous and continuous sucrose gradients as described previously (20). The clarified supernatant contained 10^7 to 10^8 50% tissue culture infective dose units of IHNV per ml.

Preparation of viral genome RNA. Purified virions were suspended in STE (0.1 M NaCl, 20 mM Tris-hydrochloride [pH 7.4], 1 mM EDTA). Self-digested pronase and sodium dodecyl sulfate (SDS) were added to 1 mg/ml and 1% (vol/vol), respectively, and the mixture was incubated for 30 min at 37°C. RNA was isolated from disrupted virions by two extractions with STE-saturated phenol, followed by one extraction with STE-saturated chloroform-isoamyl alcohol (24:1). The final aqueous phase was made 0.3 M with potassium acetate and precipitated overnight with 2.5 volumes of ethanol. RNA was pelleted and resuspended in water, and the quantity of RNA was measured by UV absorbance at 260 nm. One liter of tissue culture fluid yielded 12 to 15 μg of IHNV genome RNA. All solutions (excluding Tris buffers) and glassware used for RNA extraction were treated with diethyl pyrocarbonate to inactivate contaminating RNases.

Preparation of intracellular RNA. Monolayers of CHSE-214 cells were infected with IHNV at a multiplicity of

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infection of 10 to establish a synchronous infection, and actinomycin D was added to 0.5 μg/ml to inhibit cellular transcription. At 6 h postinfection, [5,6-^{3}H]uridine (30 Ci/mmol; New England Nuclear Corp.) was added to 10 μCi/ml. The infection was allowed to proceed until ca. 25% of the cell monolayer exhibited a cytopathic effect (24 to 28 h postinfection), at which time the monolayers were rinsed three times with ice-cold Tris-buffered saline. A lysing solution consisting of 0.5% SDS, 250 μg of proteinase K per ml, 0.1 M NaCl, 5 mM EDTA, and 30 mM Tris-hydrochloride (pH 7.4) was added, using 4 ml/150 cm^2 flask (40). The flasks then were incubated for 1 h at 37°C. Cell lysates were then pooled, and total nucleic acid was isolated by two extractions with STE-saturated phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous phase was extracted with chloroform-isoamyl alcohol (24:1), brought to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol.

The total nucleic acid preparation was treated with DNase by incubation for 1 h at 37°C in the presence of 0.1 mg of proteinase K-treated DNase (41) per ml, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 20 mM Tris-hydrochloride (pH 7.8), and 2 mM vanadyl ribonucleoside complexes (Bethesda Research Laboratories). Reactions were stopped by the addition of EDTA to 50 mM. RNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) containing 0.1% 8-hydroxyquinoline until the dye remained yellow, indicating the complete removal of the vanadyl ribonucleoside complexes. The aqueous phase then was brought to 0.3 M potassium acetate and precipitated with 2.5 volumes of ethanol. The RNA precipitates were resuspended in water, measured spectrophotometrically for concentration and purity, and stored at −70°C.

Polyadenylated RNA was selected from the total RNA preparation by column chromatography with oligodeoxythymidylic acid-cellulose by a modified procedure of Aviv and Leder (4). RNA was suspended in 1 mM EDTA and heat denatured at 65°C for 10 min. After cooling the RNA on ice, Tris-hydrochloride (pH 7.5) and NaCl were added to 10 mM and 0.5 M, respectively. Up to 10 mg of RNA was loaded onto an 8-ml column of oligodeoxythymidylic acid-cellulose and washed extensively with binding buffer (10 mM Tris-hydrochloride [pH 7.5], 0.5 M NaCl). Bound RNA was eluted by washing with 10 mM Tris-hydrochloride (pH 7.5), and fractions containing radioactivity were pooled, brought to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. Precipitated RNA was resuspended in diethyl procarbonate-treated water, quantitated, and stored at −70°C. Diethyl pyrocarbonate was used as above to inactivate RNases in solutions and glassware.

Electrophoresis of RNA. Glyoxal treatment of RNA and subsequent electrophoresis of RNA on 1% agarose gels in 10 mM sodium-phosphate buffer (pH 7.0) was carried out as described previously (26), with the exception that the glyoxal (Fluka AG, Buchs, Switzerland) required more extensive deionization. Glyoxal was passed twice over each of five 10-ml columns of Bio-Rad AG501-X8 mixed-bed resin and stored in small portions at −20°C for single use.

Electrophoresis of RNA samples in 5 mM methyl mercuric hydroxide (Alfa Products), on 1% agarose gels containing 5 mM methyl mercuric hydroxide, was carried out as described previously (5).

Both types of agarose gels were prepared for fluorography by soaking them for 2 h with agitation in liquid autoradiography enhancer (En^{3}Hance; New England Nuclear Corp.), followed by 2 h with agitation in water. Gels were dried between filter paper and cellophane for 2 h on a gel dryer (Bio-Rad) and exposed to X-ray film (Kodak X-Omat AR) for 20 to 48 h at −70°C.

Electrophoresis of proteins. Proteins were analyzed by polyacrylamide gel electrophoresis on 10% polyacrylamide gels with a 4.75% stacking gel (18). Samples were boiled for 2 min in SDS denaturing buffer (62.5 mM Tris-hydrochloride [pH 6.8], 10% glycerol [wt/vol], 5% 2-mercaptoethanol, 2.3% SDS) before electrophoresis with a constant current of 20 mA. The Laemmli gel buffer system was used for gel and reservoir buffers (18).

After electrophoresis, gels were fixed for at least 1 h in 10% acetic acid–10% trichloroacetic acid–30% methanol. Gels were prepared for fluorography by treatment with liquid autoradiographic enhancer (En^{3}Hance, New England Nuclear Corp.), dried between two pieces of cellophane, and exposed to X-ray film as above.

Densitometer analysis of autoradiograms. Autoradiographs of electrophoretic gel profiles were scanned on a Zeineh soft laser scanning densitometer (model SL-504-XL; BioMed Instruments, Inc.). Scans were printed on an Apple personal computer, using an electrophoresis reporting integrator program. Scans were photocopied, and individual peaks were cut out and weighed. Relative peak weights and mRNA molecular weights were used to calculate the molar ratio of each mRNA band with respect to mRNA band 3, which was given the arbitrary value of 1.0. The equation used was as follows: molar ratio of mRNA X = (peak weight mRNA X/peak weight mRNA 3) × (molecular weight of mRNA 3/molecular weight of mRNA X), in which mRNA X was any one of the five mRNA bands.

Cell-free translation. Polyadenylated RNA or RNA selected by hybridization to plasmid DNA was translated in vitro in a nuclease-treated rabbit reticulocyte lysate system (Bethesda Research Laboratories) as specified by the manufacturer. Reactions were carried out in 15-μl volumes for 1 h at 30°C in the presence of [^{35}S]methionine (NEG-009A; 1166.5 Ci/mmol; New England Nuclear Corp.) Newly synthesized proteins were visualized by polyacrylamide gel electrophoresis and fluorography as described above.

Hybrid selection. Hybrid selection of individual RNA species was carried out by hybridization of the polyadenylated RNA from IHNV-infected cells to plasmid DNA immobilized on nitrocellulose filters essentially as described previously (22, 33). Plasmids carrying cDNA sequences to IHNV mRNA species are listed in Table 1. The construction and characterization of these plasmids are described in an accompanying manuscript.

Plasmids, determined by RNA blot hybridization to carry sequences from each of the five translatable mRNA species, were cleaved with the restriction enzyme *Bam*HI or *Hin*dIII. Each linearized plasmid (10 μg) was heat denatured and spotted onto separate 3-mm squares of nitrocellulose filter paper (BA 85; Schleicher & Schuell, Inc.). The filters were dried overnight and baked at 80°C in vacuo for 2 h. Each filter was hybridized with 15 μg of polyadenylated RNA from IHNV-infected CHSE-214 cells for 6 h at 52°C under the conditions specified by Maniatis et al. (22). After hybridization the filters were washed, and the RNA was released, extracted, and precipitated as described above. Precipitated RNA was rinsed twice with 70% ethanol and translated in vitro.

RESULTS

Genome molecular weight. The genome of IHNV has been estimated by sucrose gradient sedimentation to be 38S to 40S (13, 25). To obtain a more exact size estimate, the

TABLE 1. Summary of hybrid selection results

Plasmid	mRNA band[a] selected	Protein encoded
pG480	2	G_0
pN421	3	N
pN419	3	N
pM1163	4	M1
pM219	4	M2
pM2132	4	M2
pM2173	4	M2
pNV58	5	NV
pNV137	5	NV
pNV711	5	NV

[a] Determined by mRNA blot hybridizations (17).

genome RNA was extracted from purified virions and analyzed by denaturing gel electrophoresis. This RNA migrated as a single high-molecular-weight band on both glyoxal and methyl mercury gels (Fig. 1), indicating that it was intact, genome-length RNA. Although both glyoxal and methyl mercury gels are reported to fully denature RNA (5, 26), the IHNV genome RNA ran differently in the two systems when compared with VSV genome standard RNA (the VSV genome has a molecular weight of $3.82 \times 10^6 \pm 0.14 \times 10^6$) (32). Glyoxal-treated IHNV genome RNA migrated slightly faster than glyoxal-treated VSV genome RNA, indicating a molecular weight of $3.7 \times 10^6 \pm 0.05 \times 10^6$. On methyl mercury gels the same IHNV genome RNA migrated significantly faster than the VSV genome, indicating a smaller molecular weight of ca. 3.5×10^6 to 3.6×10^6. Although this discrepancy should be kept in mind, we have chosen to use the glyoxal gel size estimate.

mRNA molecular weights. Isolation of the viral mRNA species was carried out by infecting CHSE-214 cells with IHNV in the presence of actinomycin D and tritiated uridine. Polyadenylated RNA from IHNV-infected cells was resolved by both glyoxal and methyl mercury gel electrophoresis into six species of RNA (Fig. 1). The largest band of RNA comigrated with purified IHNV genome RNA and was presumed to be genome-length, positive- or negative-sense molecules involved in the replication process. When purified IHNV genome RNA was fractionated with oligodeoxythymidylic acid-cellulose, less than 2% of the RNA bound to the column. Therefore, the presence of this genome-length RNA in the polyadenylated RNA preparations does not indicate a true polyadenylic acid [poly(A)] tail, but perhaps some internal short poly(A) sequences, or carry-over of genome template molecules, hybridized to polyadenylated mRNA molecules during oligodeoxythymidylic acid-cellulose chromatography.

The five subgenomic polyadenylated RNA bands were designated IHNV mRNA 1 through 5, from largest to smallest (Fig. 1). In all preparations, mRNA 2, 3, 4, and 5 were relatively abundant, whereas mRNA 1 was present in minor, more variable quantities. The marker RNA species (VSV genome, 3.82×10^6; 28S ribosomal RNA, 1.7×10^6; 18S ribosomal RNA, 6.5×10^5) (27, 32) established a reproducibly linear plot of log molecular weight versus mobility for each glyoxal gel. This was used to determine the following values for the molecular weights of the IHNV mRNA species: mRNA 1, $2.26 \times 10^6 \pm 0.035 \times 10^6$; mRNA 2, $5.63 \times 10^5 \pm 0.08 \times 10^5$; mRNA 3, $4.84 \times 10^5 \pm 0.07 \times 10^5$; mRNA 4, $3.0 \times 10^5 \pm 0.05 \times 10^5$; and mRNA 5, $1.95 \times 10^5 \pm 0.03 \times 10^5$. These values were calculated as the average of 8 to 10 separate determinations including four different preparations of polyadenylated RNA.

Although the methyl mercury gels provided excellent resolution of the RNA species, in our hands the plots of log molecular weight versus mobility were not linear over the size range of the marker RNA species and therefore were not used to determine the molecular weights of unknown RNA species.

mRNA molar ratios. The molar ratios of each mRNA species produced intracellularly during IHNV infection were determined by densitometer analysis of the autoradiograms of several glyoxal gel profiles (Fig. 2). The areas under each peak and the molecular weights of the mRNA species were used to compute the following molar ratios, normalized to 1 mol of mRNA 3: mRNA 1, 0.02 ± 0.01; mRNA 2, 0.49 ± 0.03; mRNA 3, 1.00; mRNA 4, 2.52 ± 0.40; and mRNA 5, 0.41 ± 0.14.

In vitro translation of mRNA. To confirm the viral mRNA character of the polyadenylated RNA preparations, the preparations were tested for the ability to direct viral protein synthesis in vitro. Figure 3 shows a polyacrylamide gel of the proteins synthesized in an mRNA-dependent cell-free translation reaction primed with 1 μg of the polyadenylated RNA from IHNV-infected cells. Proteins were synthesized which comigrated with IHNV standard proteins N, M1, and

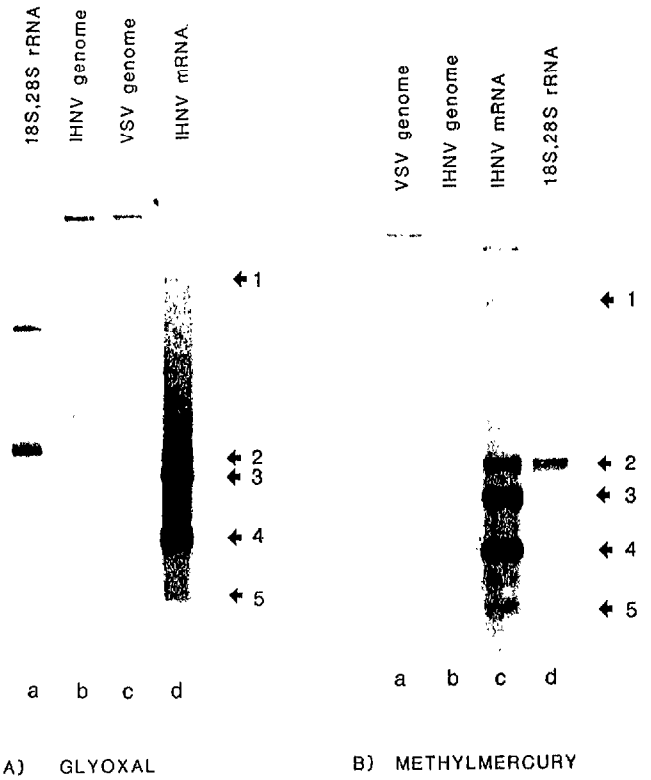

FIG. 1. Denaturing gel analyses of IHNV mRNA from infected cells. Polyadenylated RNA from IHNV-infected cells was analyzed by electrophoresis under denaturing conditions. (A) Glyoxal gel electrophoresis: a, ribosomal RNA markers (18S and 28S); b, IHNV genomic RNA extracted from purified virions; c, VSV genome marker RNA; d. polyadenylated RNA from IHNV-infected cells. (B) Methyl mercury gel electrophoresis: a, VSV genome marker RNA; b. IHNV genomic RNA from purified virions; c, polyadenylated RNA from IHNV infected cells; d, ribosomal marker RNA (18S and 28S). The number designations for the major species of mRNA are shown on each gel.

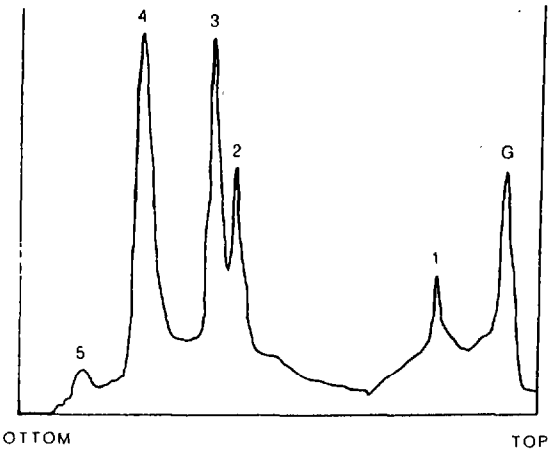

FIG. 2. Densitometer scan of the glyoxal gel profile of IHNV mRNA from infected cells. Gel profiles such as those shown in Fig. 1 were scanned with a soft laser scanning densitometer and printed with a personal computer, using an electrophoresis integration program. The mRNA band number corresponding to each peak is indicated at the top. The letter G indicates IHNV genomic RNA.

M2. A fourth protein was synthesized which migrated faster than the G protein and had a molecular weight of 56,000. This corresponds with the molecular weight of the unglycosylated G protein, G_0 (the molecular weight of G_0 is 55,000 ± 3,000; Y. Hsu, personal communication). The viral L protein was never observed in the in vitro translation products. A fifth protein with a molecular weight of 12,000 also was synthesized in vitro. Although this protein does not correspond to any of the proteins in purified virions, there is a protein of this size induced in relatively high quantities in IHNV-infected cells (Fig. 3). We have designated this the NV protein due to its nonvirion nature.

mRNA coding assignments. It was necessary to correlate which viral mRNA encoded each of the viral proteins. This was done by hybrid selection of individual mRNA species from the mixture with cloned plasmids carrying partial cDNA sequences from each viral mRNA. The construction and characterization of these clones is described elsewhere (17). In vitro translation of the mRNA selected by hybridization with each plasmid then identified the protein encoded by that mRNA.

In this way the protein coding assignments were made for all the mRNA species which could be translated in vitro. Examples for each protein are shown (Fig. 4), and a summary of the hybrid selections carried out is presented (Table 1). The plasmid which carried cDNA to mRNA band 2 hybrid selected mRNA which translated into the G_0 protein. Two plasmids which carried cDNA to band 3 selected the mRNA which coded for the N protein. Of four plasmids which carried cDNA to mRNA band 4, three selected an mRNA which coded for the M2 protein, and one selected the mRNA for the M1 protein. All three plasmids which carried cDNA to mRNA band 5 hybrid selected mRNA which translated into the small-molecular-weight NV protein.

DISCUSSION

The mRNA species of IHNV have been isolated and characterized as to molecular weights, molar ratios, and coding assignments (Table 2).

The molecular weights of the six IHNV mRNA species were used to estimate their coding capacities, after subtracting the molecular weight of a 100-residue poly(A) tail (Table 2). Our estimate of 100 residues was based on data for the poly(A) tails of VSV mRNA species (64 to 125 residues) (37) and of rabies virus mRNA species (100 to 250 residues) (14). Table 2 shows the estimated coding capacity of each IHNV mRNA, along with the actual size of the protein encoded. In each case the size of the mRNA species is more than sufficient to code for its assigned protein product. The molecular weight of 150,000 for the IHNV L protein is likely to be an underestimate by analogy with the L protein of VSV. The complete sequence of the VSV L mRNA indicates that the true molecular weight of the VSV L protein is 241,000 (38). This is significantly higher than values determined by SDS-polyacrylamide gel electrophoresis, which ranged from 160,000 to 230,000 (38). In like manner, the true molecular weight of the IHNV L protein may be higher than the value determined by gel migration and may be closer to the estimated coding capacity of mRNA 1 (Table 2). The sum of the molecular weights of the six mRNA coding sequences [excluding 100 residue poly(A) tails] is 3.9×10^6. Thus, these six genes account for 105% of the IHNV genome length, which is 3.7×10^6 (this paper).

The molar ratios of the viral mRNA species produced intracellularly during infection are shown in Table 2. The

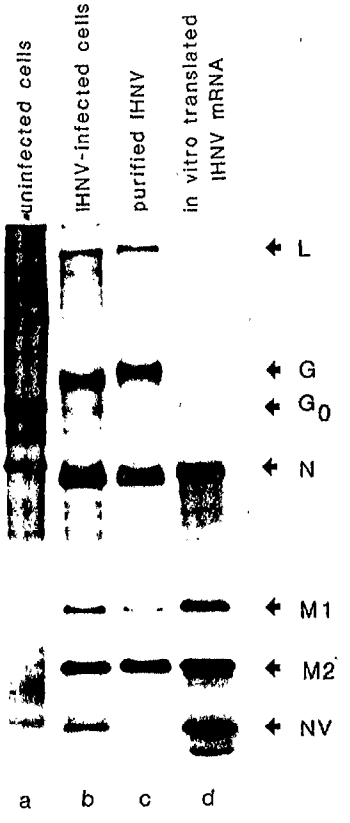

FIG. 3. Electrophoretic analyses of proteins translated in vitro from IHNV mRNA produced in infected cells. Polyadenylated RNA from IHNV-infected cells was used to prime an in vitro translation system, and the proteins produced were analyzed by SDS-polyacrylamide gel electrophoresis along with IHNV protein standards. Lanes: a, proteins from uninfected CHSE-214 cells; b, proteins from IHNV-infected cells; c, proteins from purified IHNV virions; d, proteins translated in vitro from IHNV mRNA from infected cells. The nomenclature of the IHNV proteins is indicated on the right.

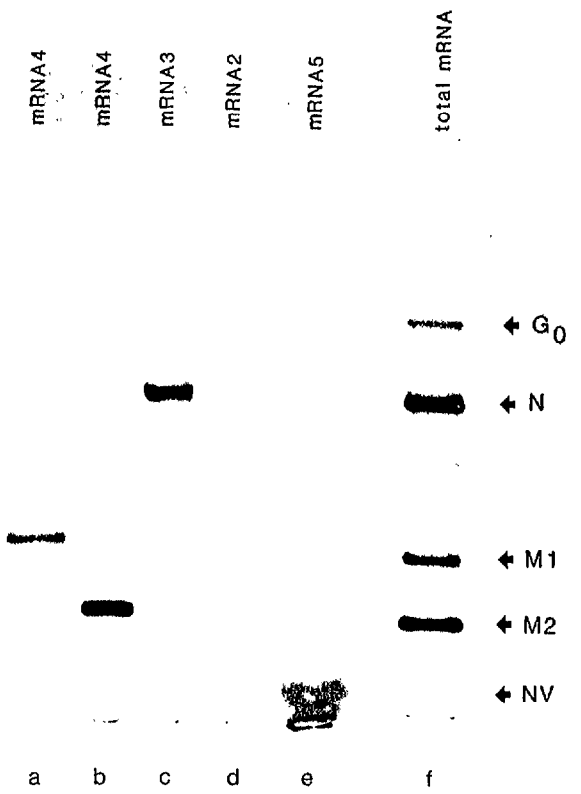

FIG. 4. Electrophoretic analysis of proteins translated in vitro from individual IHNV mRNA species after hybrid selection. Protein was translated from individual mRNA species selected from polyadenylated RNA from IHNV-infected cells by hybridization with the following cDNA plasmid: a, pM1163; b, pM219; c, pN144; d, pG480; e, pNV58. The mRNA band selected by each plasmid is indicated at the top of each lane. Lane f is a marker lane of IHNV proteins translated in vitro from the total mRNA preparation.

ratios for mRNA species 2, 3, and 4 were very consistent among different preparations of polyadenylated RNA, whereas those for mRNA species 1 and 5 were more variable. The molar ratio calculated for mRNA 1 is most likely lower than the actual value due to the increased probability of degradation for such a large mRNA (17). Since the M1 and M2 mRNA species comigrate in the gel, their individual ratios cannot be distinguished. However, the combined ratio of 2.52 indicates that one or both of these mRNA species are present in molar quantities greater than the N mRNA, which was given the arbitrary molar ratio of 1.0.

The gene order on the IHNV genome has been determined by R-loop mapping to be (3′)N-M1-M2-G-NV-L(5′) (17). With the exception of the NV gene, this is identical to the genetic maps of VSV (3′)N-NS-M-G-L(5′) (1, 6) and rabies virus (3′)N-M1-M2-G-L(5′) (10). In VSV, transcription has been shown to proceed sequentially from the 3′ to the 5′ end of the genome, with no gene transcribed until the transcription of its 3′ neighboring gene is complete (1, 6). In addition, studies with VSV have shown an attenuation of transcription at each gene junction, resulting in a decrease in the molar quantities of mRNA transcribed from each gene from the 3′ to the 5′ end of the genome (15, 42). If IHNV transcription also proceeds from the 3′ to the 5′ end along the genome, then the increase in the molar ratio of transcription from the N to the M1 or M2 genes, or both, may suggest a lack of the attenuation phenomenon. However, 5′ to the M2 gene sequential attenuation could occur, since the G, NV, and L mRNA species, respectively, are found in decreasing molar quantities (Table 2). Alternatively, the individual mRNA species may have unequal rates of turnover, in which case the observed molar ratios could not be used to assess sequential transcription or attenuation phenomena.

Although five bands of mRNA were resolved by electrophoresis (Fig. 1), there was not a one-to-one correspondence among these mRNA bands and the five known viral proteins. Instead, it was found that the mRNA species for two viral proteins comigrated in mRNA band 4, and mRNA band 5 encoded a previously undescribed viral protein.

The viral polymerase protein, L, was never observed as an in vitro translation product, presumably due to its large size (molecular weight, 150,000) (19) and the difficulty of translating large mRNA species in in vitro systems. However, there is evidence to indicate that mRNA 1 encodes the L protein, even though this could not be proven directly by hybrid selection and in vitro translation. First, cloning data confirms that mRNA 1 is of viral origin by the use of plasmids carrying cDNA to mRNA 1 (17). These cDNA inserts were shown to be viral sequences by hybridization with a cDNA probe to the viral genome and by heteroduplex formation with the viral genome. Second, the large molecular weight of the L protein (150,000) means that its mRNA must have a minimum molecular weight of 1.39×10^6. Thus, mRNA 1 is the only viral mRNA of sufficient size to encode the L protein (Table 2), in addition to being the only mRNA not accounted for by other viral proteins (Table 1).

Coding assignments for the four smaller mRNA bands were made by hybrid selection of individual mRNA species, followed by in vitro translation (Fig. 4, Table 1). IHNV mRNA bands 2 and 3 encoded the viral G_0 and N proteins, respectively. Hybrid selection with different plasmids carrying cDNA to mRNA band 4 showed the synthesis of the M1 or M2 protein, but not both. Thus, it was proven that mRNA band 4 contained two comigrating mRNA species which code for the virion M1 and M2 proteins. This is analogous to the situation in VSV, in which the fourth mRNA band visible on denaturing polyacrylamide gels includes both the NS and M mRNA species (16, 37).

Thus, all five of the known virion proteins were accounted for by mRNA species 1 through 4, and the identity of mRNA 5 remained to be determined. Proof that mRNA 5 was of viral origin came again from the cloning data (17). Three plasmids were isolated which carried cDNA to mRNA 5. The cDNA inserts of these plasmids hybridized with a cDNA probe to the viral genome and also formed heteroduplexes with the viral genome. Thus, mRNA 5 is coded for by the IHNV genome and is not a cellular mRNA.

TABLE 2. Characteristics of IHNV mRNA species

IHNV nRNA	Mol wt	Molar ratio	Estimated coding capacity[a]	Protein encoded	Protein[b] mol wt
1	2.26×10^6	0.02	240,000	L	150,000
2	5.63×10^5	0.49	57,000	G_0	55,000
3	4.48×10^5	1.0	48,500	N	40,500
4	3.00×10^5	2.52	28,700	M1	22,500
				M2	17,000
5	1.95×10^5	0.41	17,400	NV	12,000

[a] Calculated, assuming a poly(A) tail of 100 residues (14, 37).
[b] Determined by SDS-polyacrylamide gel electrophoresis (19).

Hybrid selection in which the plasmids with cDNA to mRNA 5 were used resulted in the synthesis of a protein with a molecular weight of 12,000. Electrophoretic profiles of purified IHN virions labeled with [^{35}S]methionine confirmed that this protein is not present in mature virions (Fig. 3). However, IHNV-infected cell proteins labeled with [^{35}S]methionine did show the induction of substantial amounts of a 12,000-molecular-weight protein. Since the function of this protein remains to be determined, it was designated the NV protein, due to its nonvirion nature. The NV protein translated from hybrid-selected mRNA 5 appeared on gels as a more diffuse band compared with the intact band produced during in vitro translation of the whole polyadenylated RNA preparation (Fig. 3 and 4). This may be due to some alteration or preferential degradation of this small mRNA during the manipulations of hybrid selection.

Hybrid-selected preparations of individual mRNA species were sometimes contaminated with trace amounts of mRNA for other proteins, as shown by the faint N and M2 protein bands in lanes a and d (Fig. 4). This may be due to some regions of homology between these mRNA species or simply to the high relative abundance of the N and M2 mRNA species. Nevertheless, in each case a single viral protein was reproducibly the major species translated from hybrid-selected mRNA, enabling us to make conclusive coding assignments which are summarized in Table 1.

To our knowledge, the NV protein is the first nonvirion viral protein discovered in a rhabdovirus. Viral proteins synthesized in infected cells but not assembled into mature virions have been reported for positive-strand RNA viruses such as picornaviruses and togaviruses, in which the function(s) proposed for the nonvirion proteins is generally related to an RNA replicase activity (21). However, IHNV, like all rhabdoviruses, carries a viral polymerase in the virion. This polymerase is capable of carrying out the transcription of viral mRNA species in in vitro reactions containing lysed purified virions (24; G. Kurath and J. C. Leong, manuscript in preparation). Since these reactions would not contain any NV protein, it appears that NV protein is not necessary for viral transcription. It may be involved with viral replication or influence host cell processes, both of which occur in the infected cells.

With the exception of the NV protein, IHNV resembles VSV and rabies virus very closely with respect to its proteins, mRNA species, genome size, and genome organization. Thus, we are lead to question whether there might be a nonvirion protein analogous to the IHNV NV protein associated with VSV, rabies, or both. The small molecular weight of the NV protein (12,000) and its mRNA (1.95×10^5; ca. 570 bases) mean that the coding sequence on the genome would be only 370 to 470 bases, depending on the length of the NV mRNA poly(A) tail (14, 37). Classical genetics with VSV mutants indicate the possible existence of six complementation groups in both the Indians and New Jersey serotypes (9, 31). This suggests that there are six viral genes rather than five. However, cloning and sequencing studies with VSV now have yielded the nucleotide sequence of the entire VSV genome, and there is no indication of a sixth gene (11, 34–36, 38). In addition, the original characterizations of the mRNA species of VSV and rabies virus did not show any mRNA species which were not accounted for by the virion proteins (7, 8, 16, 29, 37, 39).

If there is a nonvirion protein analogous to the IHNV NV protein in VSV and other rhabdoviruses, then this protein may have a general role in the rhabdovirus replication cycle. Alternatively, if VSV and rabies virus have no analog to the NV protein, it may be that the role of this protein is somehow specific to replication in the fish cells of its host or to replication at colder temperatures. The optimum temperature for IHNV replication is 16°C, and the virus is inactivated at 20°C (30). It would be of interest to determine whether a protein analogous to the NV protein is coded for by other piscine rhabdoviruses such as spring viremia of carp virus, pike fry rhabdovirus, or viral hemorrhagic septicemia virus (30).

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MANUSCRIPT # 2

Molecular Cloning of the Six mRNA Species of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus, and Gene Order Determination by R-Loop Mapping

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Molecular Cloning of the Six mRNA Species of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus, and Gene Order Determination by R-Loop Mapping†

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Plasmids carrying cDNA sequences to the mRNA species of infectious hematopoietic necrosis virus were constructed and cloned into *Escherichia coli*. Characterization of 21 cloned plasmids by hybridization to mRNA blots identified sets of plasmids with homology to each of the six viral mRNA species. R-loop mapping with these cDNA plasmids determined that the gene order on the infectious hematopoietic necrosis virus genome is (3′)N-M1-M2-G-NV-L(5′).

Infectious hematopoietic necrosis virus (IHNV) is a salmonid rhabdovirus with a single-stranded RNA genome of ca. 10,900 bases (11). The virion is comprised of five proteins (9, 14, 19): a nucleocapsid protein, N; a surface glycoprotein, G; two matrix proteins, M1 and M2; and a polymerase, L. Although IHNV resembles the mammalian rhabdovirus prototypes, vesicular stomatitis virus, and rabies, there are some significant differences, including the encoding of a nonvirion protein which has been designated the NV protein (11).

The slow growth and relatively low yield of IHNV in tissue culture (15) made it necessary to obtain cloned genetic material for further studies of the molecular biology of the virus. We present here the construction of cDNA clones carrying sequences from each of the six mRNA species of IHNV. These clones were used in hybrid selection studies to determine coding assignments for the viral mRNA species (11) and in R-loop analyses to obtain a physical map of the viral genome.

MATERIALS AND METHODS

Cells and virus. IHNV was propagated in a chinook salmon embryo cell line (CHSE-214) as described previously (11).

Cloning of viral mRNA species. Polyadenylated RNA was isolated from IHNV-infected CHSE-214 cells as described previously (11). For use as a cloning template, this RNA was passed twice over an oligodeoxythymidylic acid-cellulose column to remove all detectable host cell ribosomal RNA. The preparation of double-stranded cDNA was carried out by the procedure of Land et al. (13). This procedure includes 4 mM sodium PP_i in the first-strand cDNA reaction to prevent the formation of the terminal hairpin loop and eliminates the need for S1 nuclease digestion.

Briefly, 20 μg of polyadenylated RNA was reverse transcribed to synthesize 3.9 μg of single-stranded cDNA in a reaction containing the RNA template, an oligodeoxythymidylic $acid_{(12-18)}$ primer, placental RNase inhibitor (Enzo Biochemicals, Inc.), and reverse transcriptase (Life Science Div., The Mogul Corp.). Tails of ca. 20 dCMP residues were added to this single-stranded cDNA with terminal deoxynucleotidyl transferase, and these molecules were rendered double stranded in a second reverse transcriptase reaction containing oligodeoxyguanylic $acid_{(12-18)}$ (Collaborative Research, Inc.) as the primer. Nicks or gaps in these double-stranded molecules were filled by incubation with Klenow enzyme (18), and the products of this reaction were tailed with dCMP residues.

The plasmid vector, pUC8, was cleaved with the restriction endonuclease *Pst*I (Bethesda Research Laboratories), and deoxyguanylic acid tails of ca. 15 residues were added. The deoxyguanylic acid-tailed vector and deoxycytidylic acid-tailed cDNA were annealed at a molar ratio of 1:1 (13), and this DNA was used to transform two host strains of *Escherichia coli* K-12, JM103 (22), and C600 SC181 (2). Transformation was carried out with freshly prepared competent cells by the calcium chloride shock method (17). Transformants were plated on LB agar (18) containing 150 μg of ampicillin per ml. The transformation efficiencies were 51 and 158 ampicillin-resistant transformants per ng of reannealed DNA for JM103 and SC181, respectively. The control transformation efficiency was 5×10^3 transformants per ng of uncleaved pUC8 for both strains.

Preparation of cDNA probe. The probe for viral-specific sequences was ^{32}P-labeled cDNA to the IHNV genome RNA. Probe synthesis was carried out at 42°C for 105 min in a 50-μl reaction containing 1 μg of fragmented viral genome RNA; 0.5 μg of calf thymus random primer fragments (28); 50 mM Tris-hydrochloride (pH 8.3); 40 mM $MgCl_2$; 0.4 mM dithiothreitol; 0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP, and 0.01 mM [^{32}P]dCTP (specific activity, 800 Ci/mmol; New England Nuclear Corp.); and 40 U of reverse transcriptase (Life Science Div.). After synthesis the reaction was adjusted to 0.6 N NaOH and incubated at 37°C for 30 min. The reaction was then neutralized by adding HCl to 0.6 N and Tris-hydrochloride (pH 8.1) to 200 mM. This mixture was passed over a 5-ml column of Sephadex G-50, and fractions containing incorporated radioactivity were pooled, adjusted to 0.3 M potassium acetate, and precipitated with 2.5 volumes of ethanol. This probe typically had a specific activity of 2×10^7 cpm/μg.

Colony blots. Transformants were screened for viral sequences by the procedure of Taub and Thompson (27), in which fresh colonies were replicated onto sterile Whatman 541 filter paper and washed successively in NaOH, lysozyme, proteinase K, and phenol-chloroform-isoamyl alcohol (25:41:1). Filters were hybridized with 2×10^6 to 5×10^6

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cpm of ^{32}P-labeled cDNA probe (see above) as described previously (27).

Isolation of plasmid DNA. Small-scale plasmid preparations (1 to 2 μg) were made by a modification of the alkaline lysis procedure (4). Fresh colonies of each transformant were scraped from plates with a toothpick and dispersed vigorously in 40 μl of a lysing buffer composed of 0.5% sodium dodecyl sulfate, 50 mM NaOH, 5 mM EDTA, and 2% Ficoll. These mixtures were incubated for 30 min at 68°C. Sucrose and bromphenol blue were added to 5 and 0.002%, respectively, and the samples were loaded onto a horizontal 0.7% agarose gel in Tris-acetate buffer (0.72 M Tris-hydrochloride, 0.1 mM acetate, 20 mM EDTA, [pH 7.9]). After electrophoresis for 12 to 16 h at 25 V, the gel was stained with ethidium bromide and examined with a UV transilluminator (Fotodyne, Inc.).

Large-scale isolation of plasmid DNA was carried out by the boiling method of Holmes and Quigley (10).

Determination of cloned viral sequence size. The restriction endonuclease *Pst*I (Bethesda Research Laboratories) was used to cleave 300 ng of each purified plasmid. The released cloned insert DNA was separated from the plasmid vector DNA by electrophoresis on a vertical 7.5% acrylamide gel with a 3.75% acrylamide stacking gel. The Laemmli gel system (12) was used with the exception that sodium dodecyl sulfate was omitted from all buffers. Electrophoresis was carried out at 30 mA through the stacking gel and 50 mA through the lower gel. After electrophoresis, the gel was stained with ethidium bromide, and photographs of the UV-illuminated gel were made with Polaroid type 47 film. The sizes of the cloned inserts were determined by comparison with *Hin*fI and *Hae*III pBR322 restriction fragment size standards.

Preparation of nick-translated probes. Nick translation of cloned plasmids was carried out as described previously (18, 24) in 10-μl reactions containing 200 ng of plasmid DNA. Nick-translated probes had a specific activity of 2×10^7 to 5×10^7 cpm per μg.

DNA blot hybridizations. Cloned plasmids were cleaved with the restriction endonuclease *Pst*I (Bethesda Research Laboratories), and the cloned inserts were separated from vector DNA by electrophoresis on 0.7% agarose gels in Tris-acetate buffer (see above). The DNA was alkaline denatured, transferred to a nitrocellulose membrane by the Southern blot method (18, 26), and baked onto the filter for 2 h at 80°C in vacuo. Hybridization of the blots was as described previously (18) with the exception that both prehybridization and hybridization were carried out in 50% formamide at 42°C. Each hybridization included 2×10^6 to 10×10^6 cpm of ^{32}P-labeled cDNA probe or nick-translated probe. After hybridization, blots were washed as described previously (18) and exposed to Kodak X-Omat AR X-ray film with a Cronex Lightening-Plus intensifying screen (Du Pont Co.) at −70°C.

mRNA blot hybridization. Hybridization of ^{32}P-labeled, nick-translated probes to blots of viral mRNA were carried out by the procedures of Thomas (29). Polyadenylated RNA from IHNV-infected cells was resolved into five bands by glyoxal gel electrophoresis (11, 21) and transferred to a nitrocellulose membrane as described previously (29). The blot was baked for 2 h in vacuo at 80°C and cut into strips, each containing one gel lane with 2 μg of RNA. The strips were boiled for 5 min in 20 mM Tris-hydrochloride (pH 8.0) to remove the glyoxal adducts and hybridized individually as described previously (29) with 5×10^6 to 10×10^6 cpm of ^{32}P-nick-translated probes. After hybridization, blots were washed as described previously (29) and exposed to X-ray film as described above.

R-loop mapping. IHNV genomic RNA for R-loop mapping was prepared as described previously (11). Cloned IHNV cDNA plasmids were prepared as described above for large-scale isolation and linearized by cleavage with *Nde*I (Bethesda Research Laboratories). Formamide (Bethesda Research Laboratories) was deionized by treatment with AG 501-X8 mixed bed ion-exchange resin (Bio-Rad Laboratories). R-loops were formed as previously described (5) with the following modifications. Linearized plasmid DNA (100 ng) in 18 μl of 78% formamide–5 mM EDTA was denatured at 80°C for 10 min. IHNV RNA (100 ng) was added to the denatured DNA, the solution was adjusted to 20 μl of 70% formamide–300 mM NaCl–4.5 mM EDTA, and R-loops were formed by incubation at 50°C for 12 to 16 h. Samples (4 μl) of the R-loop reaction mixture were spread by the urea-formamide method (23). The hyperphase (40 μl) consisted of 4 M urea, 80% formamide, 5 mM EDTA, and cytochrome *c* at a concentration of 40 μg/ml. The hypophase (20 ml) was 50% formamide. The DNA-protein film was adsorbed to a Parlodion-coated grid, stained with uranyl acetate, and rotary shadowed with platinum-palladium. Grids were examined with a Zeiss EM-10A electron microscope operating at 40 kV. Molecular lengths were measured by a calculator-driven digitizer on photographic prints enlarged to a final magnification of 136,000. DNA molecules of known sequence were used as length standards. Double-stranded DNA and RNA · DNA hybrid duplexes had identical contour lengths under the spreading conditions used in these experiments. IHNV RNA lengths were converted to nucleotides, using a unit length of 10,900 nucleotides and correcting for RNA in RNA · DNA hybrid duplexes.

RESULTS

Preparation of cloned plasmids carrying viral cDNA. The mRNA species of IHNV have been isolated from IHNV-infected salmon cells and resolved electrophoretically into five bands which range in size from 1.95×10^5 to 2.26×10^6 (11). This RNA was used as a template for the preparation of cDNA clones carrying IHNV mRNA sequences. Briefly, polyadenylated RNA from IHNV-infected cells was copied into cDNA with reverse transcriptase and an oligodeoxythymidylic acid primer. Analysis of the products of the first-strand cDNA reaction by alkaline gel electrophoresis showed four distinct species corresponding in size to full-length copies of the mRNA bands 2, 3, 4, and 5 (data not shown). This single-stranded cDNA was copied into double-stranded molecules, polydeoxycytidylic acid tailed with terminal transferase, and annealed into the *Pst*I site of the plasmid vector pUC8. Two *E. coli* K-12 host strains, JM103 and SC181 (C600), were transformed, and ca. 800 ampicillin-resistant transformants were isolated from each. Colony-blotted DNA of ca. 90% of the transformants hybridized with a viral genome cDNA probe. Small-scale plasmid preparations (1 to 2 μg) showed that ca. 10% of the transformants had relatively large plasmids. The cDNA inserts of purified plasmids from 21 of these transformants were released by *Pst*I cleavage and determined by electrophoresis to range in size from 155 to 640 base pairs (Table 1). Southern blot analyses of *Pst*I-cleaved plasmids showed that the insert DNA hybridized strongly with a viral genome cDNA probe (data not shown), reconfirming that the cloned sequences were virus specific.

mRNA blot analyses. Characterization of the cloned plasmids required that we identify the specific viral mRNA

TABLE 1. Viral insert size and mRNA specificity for cloned plasmids

Cloned plasmid	Insert size (base pairs)	mRNA specificity[a]	Viral gene[b]
pL232	640	1	L
pL262	640	1	L
pG480	440	2	G
pN512	370	3	N
pN933	320	3	N
pN421	350	3	N
pN154	405	3	N
pN156	400	3	N
pN419	460	3	N
pN125	450	3	N
pN144	450	3	N
pM1163	425	4	M1
pM1420	155	4	M1
pM219	505	4	M2
pM2173	265	4	M2
pM211	510	4	M2
pM2112	515	4	M2
pM2132	510	4	M2
pNV58	420	5	NV
pNV137	395	5	NV
pNV711	220	5	NV

[a] Determined by mRNA blot hybridizations (Fig. 1).
[b] Determined for all genes but L by hybrid selection and in vitro translation. The L gene was assigned tentatively to mRNA 1 by molecular weight correlation (11).

TABLE 2. Cross-hybridization within sets of plasmids with cDNA to the same viral mRNA species

Plasmids	pNV58	pL262	pN144	pN419	pM219	pM1163
A. mRNA band 5						
pNV58	+					
pNV137	+					
pNV711	+					
B. mRNA band 1						
pL262		+				
pL232		+				
C. mRNA band 3						
pN512			+	−		
pN933			+	−		
pN421			+	−		
pN154			+	−		
pN156			+	−		
pN125			+	−		
pN144			+	−		
pN419			−	+		
D. mRNA band 4						
pM219					+	−
pM2173					+	−
pM2112					+	−
pM2132					+	−
pM1163					−	+
pM1420					−	+

species which was complementary to each cDNA insert. This was carried out by probing nitrocellulose blots of the mRNA electrophoretic pattern with ^{32}P-labeled probes made by nick translation of each of the 21 purified plasmids. Examples of plasmids which hybridized specifically to each of the five mRNA bands are shown in Fig. 1.

Identification of the single mRNA band which hybridized with each plasmid DNA probe was made by comparison of the blot autoradiogram with a marker lane of all five mRNA bands (Fig. 1a). However, the proximity of mRNA bands 2 and 3 made it difficult to distinguish hybridization to mRNA band 2 with certainty. Therefore, a double hybridization was carried out with two probes together, pG480 and pN144 (Fig. 1g). The presence of two bands of hybridization confirmed that cDNA clones with sequences from both mRNA bands 2 and 3 had been isolated (Table 1). Of the 21 plasmids tested,

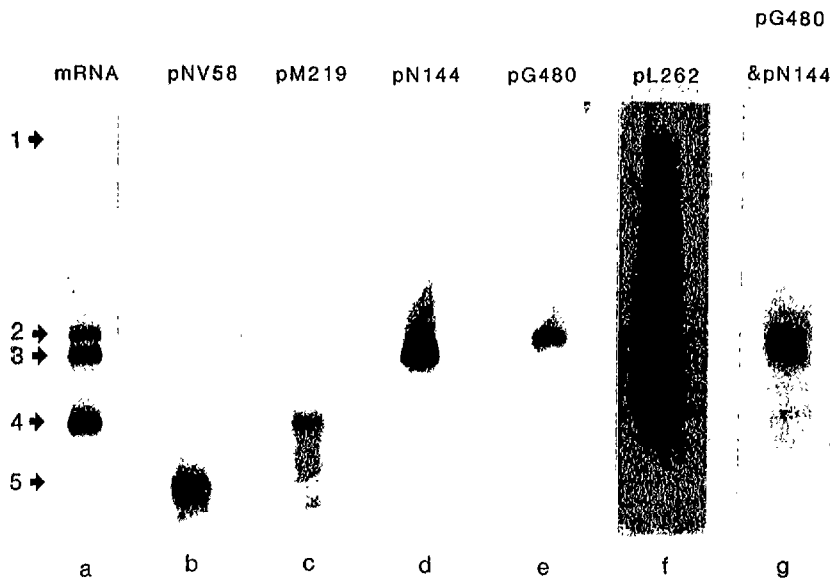

FIG. 1. mRNA blot hybridization analyses of cloned plasmids. IHNV mRNA isolated from infected salmon cells (11) was resolved by glyoxal gel electrophoresis, blotted onto a nitrocellulose membrane, and hybridized with ^{32}P-labeled probes made by nick translation of cloned plasmids. Lane a is an autoradiogram of the IHNV ^{3}H-mRNA electrophoretic profile identical to those used to prepare the blots. Numbers to the left of lane a designate IHNV mRNA bands 1 through 5. Lanes b through g are autoradiograms of blots after hybridization with probes of the following plasmids; b, pNV58; c, pM219; d, pN144; e, pG480; f, pL262; g, pG480 and pN144.

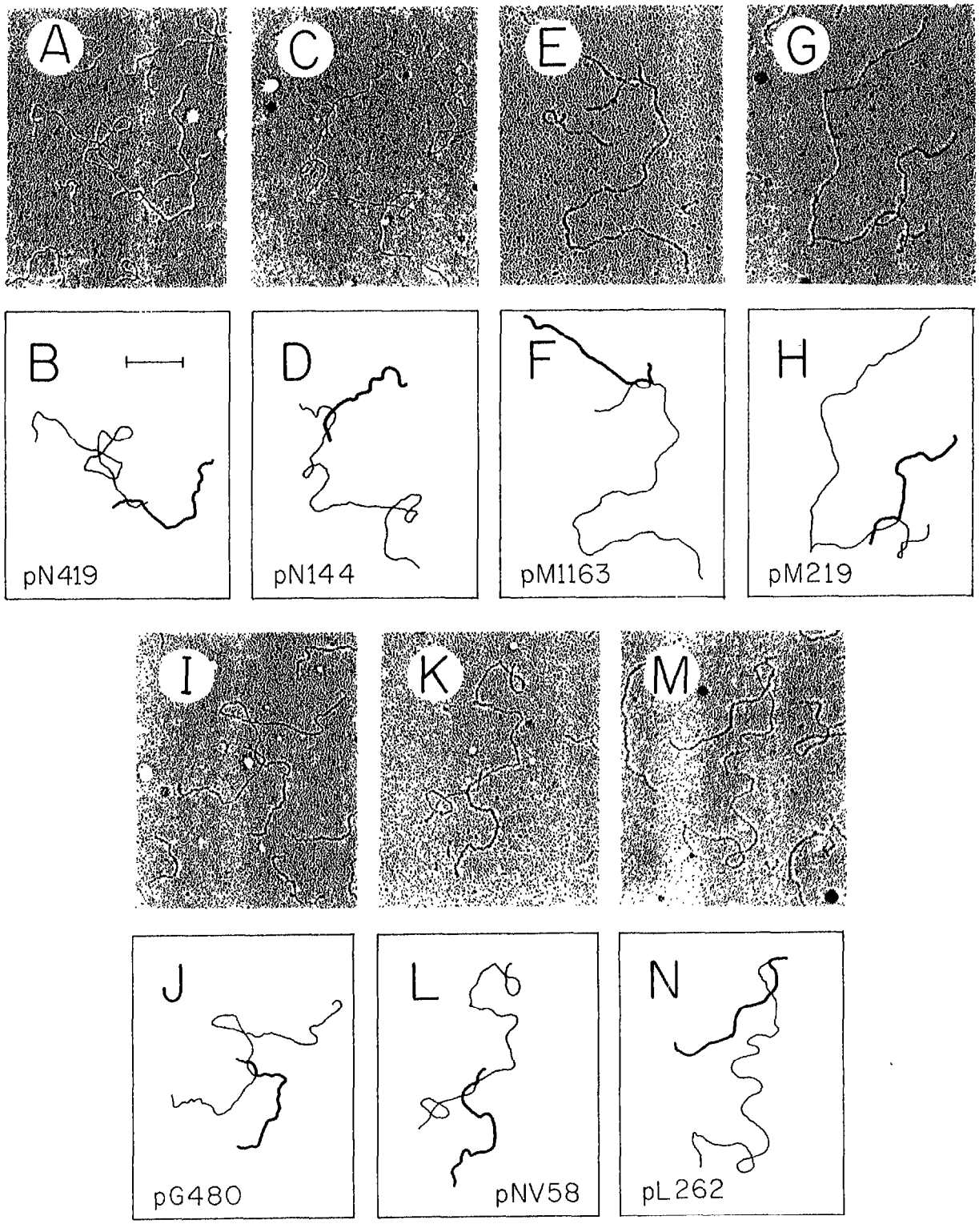

FIG. 2. Analysis by electron microscopy of R-loops formed between IHNV genomic RNA and plasmids containing cloned IHNV cDNA sequences. Interpretive drawings of each R-loop are shown below each photograph in which the thin line is IHNV RNA (or the displaced, single-stranded DNA in the R-loop) and the thick line is double-stranded plasmid DNA (or the RNA · DNA hybrid duplex in the R-loop). The bar in panel B represents 1,000 base pairs. R-loops were formed between IHNV RNA and pN419 (A and B), pN144 (C and D), pN1163 (E and F), pM219 (G and H), pG480 (I and J), pNV58 (K and L), and pL262 (M and N).

TABLE 3. Measurements of single R-loops and location of hybridizations on the viral genome

Plasmid	Measurements of[a]:			Location of[b]:	
	Short end	Loop	Long end	N-ward nucleotide	L-ward nucleotide
pN419		490 ± 50	10,410 ± 500		490 ± 50
pN144	960 ± 110	440 ± 40	9,500 ± 450	960 ± 110	1,400 ± 150
pM1163	1,520 ± 220	410 ± 60	8,970 ± 390	1,520 ± 220	1,930 ± 285
pM219	2,020 ± 250	530 ± 60	8,350 ± 470	2,020 ± 250	2,550 ± 310
pG480	4,180 ± 190	400 ± 40	6,320 ± 240	4,180 ± 190	4,580 ± 230
pNV58	4,430 ± 240	450 ± 40	6,020 ± 270	4,430 ± 240	4,880 ± 280
pL262		710 ± 70	10,190 ± 500	10,190 ± 500	10,900 ± 570

[a] In nucleotides (± standard deviation) assuming 10,900 nucleotides as the complete length of the genome.
[b] Nucleotide position (± standard deviation) of R-loop boundaries from 0 (N-ward end) to 10,900 (L-ward end).

2 hybridized to mRNA 1, 1 hybridized to mRNA 2, 8 hybridized to mRNA 3, 7 hybridized to mRNA 4, and 3 hybridized to mRNA 5.

Cross-hybridization studies. It was of interest to examine the extent of cross hybridization between the cDNA inserts of these cloned plasmids. This was done by hybridizing blots of *Pst*I-restricted, electrophoretically resolved plasmid DNA with probes made by nick translation of other cloned plasmids. As was expected, there was strong hybridization of every probe to the linear pUC8 DNA. However, hybridization to the released insert DNA was dependent on the specific cDNA of each probe. In no case was there hybridization between inserts which had been identified previously as carrying cDNA to different mRNA bands (data not shown).

Within sets of plasmids which hybridized to the same mRNA band there was considerable homology. Salient results of the cross-hybridization studies are compiled in Table 2. The inserts of the three plasmids which carried cDNA to mRNA band 5 all showed specific cross-hybridization. In like manner, the inserts of the two mRNA band 1 plasmids hybridized to each other. Of the eight plasmids with cDNA to mRNA band 3, seven of the inserts did cross-hybridize, and one insert, pN419, did not. Hybridization selection studies (11) have shown that both pN419 and the other mRNA band 3 plasmids are complementary to the mRNA for the viral N protein.

Analysis of the seven plasmids which hybridized to mRNA band 4 indicated that there were two mutually exclusive subsets of homologous cDNA plasmids (Table 2D). Hybrid selection studies (11) revealed that mRNA band 4 was comprised of two distinct comigrating mRNA species which encode the viral M1 and M2 proteins. A subset of five cross-hybridizing mRNA 4 plasmids carried cDNA to the mRNA for M2, and a subset of two mRNA 4 plasmids carried cDNA to the mRNA for M1 (Table 1).

Heteroduplex mapping. Once cDNA clones were identified which carried sequences from each of the six viral genes, it was possible to determine the physical order of the genes on the viral genome by R-loop mapping with viral genomic RNA.

The first set of R-loops was prepared by annealing viral genomic RNA with a single cloned plasmid carrying cDNA to each viral gene. Plasmids pL262, pG480, pN144, pM1163, pM219, and pNV58 were used to locate genome sequences of the L, G, N, M1, M2, and NV genes, respectively. In addition, plasmid pN419 was included since it failed to cross-hybridize with the other plasmids carrying cDNA from mRNA 4 (Table 2C). Examples of the R-loops observed are shown in Fig. 2. Measurements of 15 to 20 R-loops formed with each plasmid were used to locate the region of hybridization along the viral genome. The percentage of the full-length genome RNA on both sides of each R-loop was determined and converted to nucleotide values, assuming 10,900 bases as the full length of the genome (11) (Table 3).

Both of the inserts of the N gene plasmids, pN144 and pN419, hybridized near the end of the genome, within the terminal 10% of the complete genome length. The L gene plasmid, pL262, also hybridized within the terminal 7% of the genome length. Since the L and N insert sequences did not cross-hybridize, we concluded that these clones were hybridizing to opposite ends of the genome. Thus, we were able to orient the genome with respect to N-ward and L-ward ends.

These data alone were not sufficient to determine the gene order because the two ends of the genome were indistinguishable in the electron micrographs of single R-loops. Therefore, to determine the relative positions of the viral genes, double R-loop reactions were carried out in which pairs of cloned plasmids were annealed simultaneously to genomic RNA. Examples of genomic RNA with two R-loops are shown in Fig. 3, and appropriate measurements are given in Table 4. The distances between pairs of R-loops formed between the pN144 and pM1163, mP219, pG489, or pNV58 indicate that the N, M1, M2, G, and NV genes are clustered, in that order, within the N-ward half of the genome. The long distance between double R-loops formed with pL262 and pM1163 indicated that the L gene insert did hybridize to the end of the genome distal from the other five genes. Thus, the order of the genes from the N-ward to the L-ward ends of the genome was concluded to be N-M1-M2-G-NV-L.

DISCUSSION

We have presented the construction and characterization of cDNA clones carrying sequences from each of the six mRNA species of IHNV. The strategy for constructing the clones involved the use of an oligodeoxythymidylic acid primer, which would hybridize to the 3′ polyadenylated tail of the viral mRNA species and prime cDNA synthesis in the 5′ direction. Theoretically, all the cloned plasmids should contain the intact 3′ terminal sequence of the corresponding viral mRNA and various lengths of sequence in the 5′ direction. Although alkaline gel analysis of the first-strand cDNA products indicated the synthesis of complete copies of mRNA bands 2, 3, 4, and 5 (data not shown), there were no full-length sequences found upon analysis of the cloned plasmids. We hypothesize that this occurred because of the lack of size selection before annealing and the preferential annealing of shorter double-stranded cDNA molecules into the vector. Thus, the clones we have described carry partial sequences of each viral mRNA and should include the 3′-terminal sequences.

The plasmids from a set of 21 transformants were characterized by hybridization to blots of viral mRNA, and the

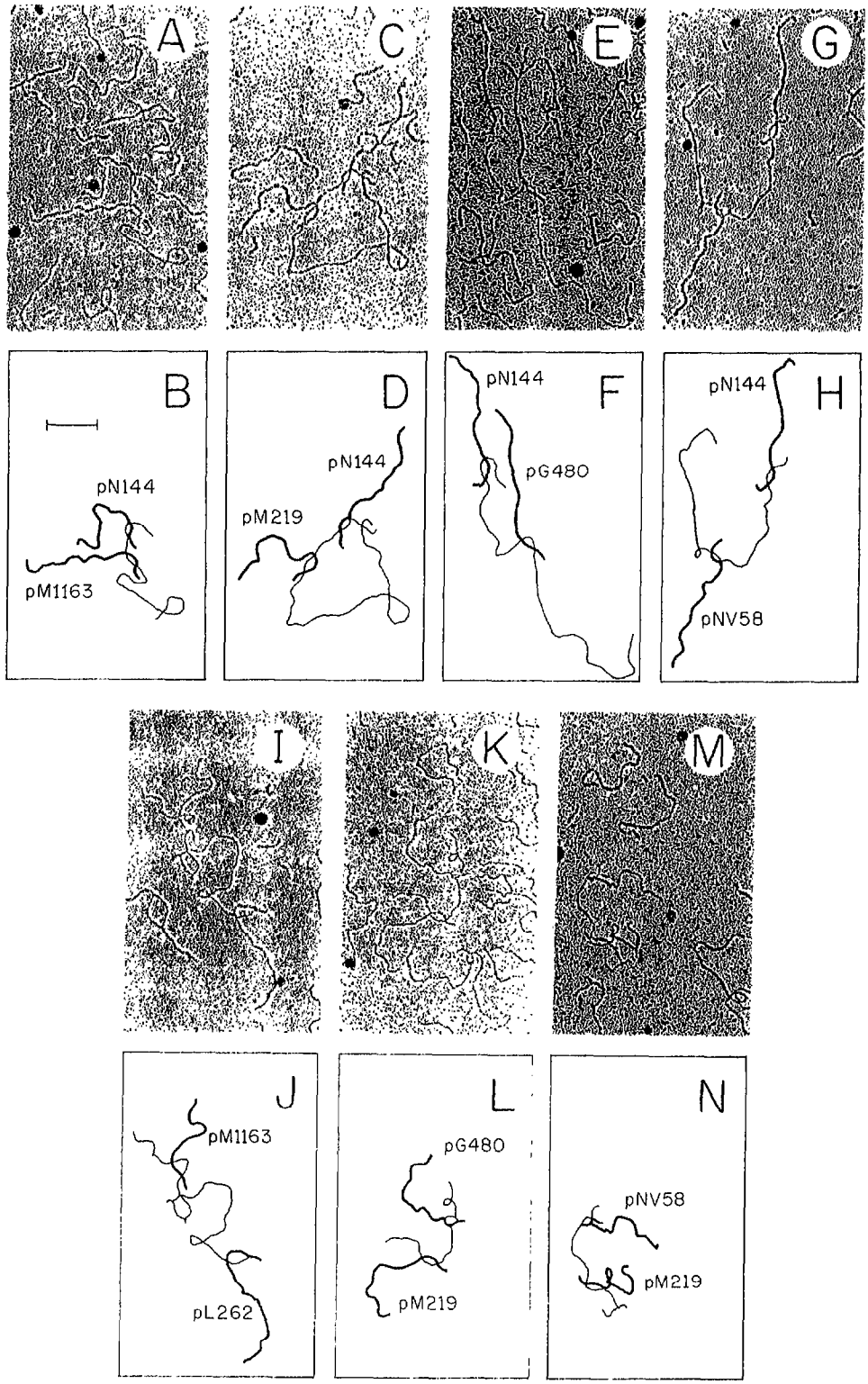

FIG. 3. Analysis by electron microscopy of R-loops formed between IHNV genomic RNA and pairs of plasmids containing cloned IHNV cDNA sequences. See the legend to Fig. 2 for details. The bar in panel B represents 1,000 base pairs. R-loops were formed between IHNV RNA and the following pairs of plasmids: pN144 and pM1163 (A and B), pN144 and pM219 (C and D), pN144 and pG480 (E and F), pN144 and pNV58 (G and H), pM1163 and pL262 (I and J), pM219 and pG480 (K and L), and pM219 and pNV58 (M and N).

TABLE 4. Gap measurements for double R-loops

Plasmids	Gap[a]
pN144 and pM1163	440 ± 30
pN144 and pM219	1,140 ± 110
pN144 and pG480	2,710 ± 410
pN144 and pNV58	3,260 ± 400
pM1163 and pL262	7,630
pM219 and pG480	1,470 ± 70
pM219 and pNV58	2,160 ± 100

[a] In number of nucleotides (± standard deviation) between adjacent boundaries of the two R-loops.

specific mRNA species which was complementary to each cloned sequence was determined (Table 1). The number of clones carrying cDNA to each mRNA band generally reflected the relative abundance of that mRNA in the total mRNA preparation used as the template for cloning. Thus, the majority of the clones carried cDNA to mRNA bands 3 and 4, which had the highest molar ratios of the mRNA species in the total preparation (11).

Cross-hybridization studies showed that the majority of cloned sequences which hybridized to the same mRNA band also hybridized with each other (Table 2). This indicated that the same or significantly overlapping regions of the mRNA sequence had been cloned in most cases, as would be expected if each contained the 3′ mRNA terminus. The only exception to this was plasmid pN419, which hybridized to mRNA band 3 but did not hybridize with any of the other seven clones exhibiting hybridization to mRNA band 3 (Table 2C). R-loop mapping with these plasmids showed that pN419 hybridized to a region covering ca. 490 bases at one end of the genome. One of the other seven cross-hybridizing mRNA band 3 plasmids, pN144, hybridized at ca. 960 to 1,400 bases from the genome end (Table 3). Since the entire length of mRNA 3 is estimated to be 1,420 bases (molecular weight, 4.84×10^5; 11), these hybridization measurements indicate that these two plasmids contain nonoverlapping sequences from the two ends of the N gene. If, as the cloning strategy predicts, the majority of the clones carry the 3′ sequences of the mRNAs, then pN144, representing the seven cross-hybridizing plasmids, contains the 3′ sequences of mRNA 3. The insert of pN419 would then contain the 5′ mRNA band 3 sequences. This could conceivably have originated from a full-length, single-stranded cDNA molecule which, upon 5′ priming, carried out incomplete second-strand synthesis. An analogous clone containing 5′ sequences of the N mRNA has been reported for vesicular stomatitis virus (25).

Examination of the R-loops of pN419 and the viral genome revealed a very short length of unhybridized terminal genomic RNA (Fig. 3A). This may be indicative of a short terminal leader sequence next to the N gene, as in vesicular stomatitis virus (6, 7, 16).

The gene order on the IHNV genome was determined by double R-loop mapping to be N-M1-M2-G-NV-L (Table 4, Fig. 4). With the exception of the NV gene, this order is identical to that of the analogous genes of vesicular stomatitis virus (3′)N-NS-M-G-L (5′) (1, 3) and rabies (3′)N-M1-M2-G-L(5′) (8). Thus, although no 3′ to 5′ orientation was determined directly for the IHNV genome, we assume that the N-ward end is 3′, since an exact inversion of gene order for this rhabdovirus is highly unlikely. In addition, assuming that the cDNA clones contain the 3′ mRNA sequences (with the exception of pN419), then the position of each R-loop agrees with the 3′N to L5′ orientation (Fig. 4). In particular, the hybridization of the pL262 insert to the extreme terminus of the genome, rather than in the middle, implies that the L terminus is the 3′ end of the mRNA sequence, and thus the 5′ end of the gene and genome.

The size of each gene was estimated from the R-loop measurements by assuming that the 5′ boundaries of the R-loops of pN144, pM1163, pM219, pG480, and pL262 corresponded to the 5′ ends of the N, M1, M2, G, NV, and L genes, respectively. These gene sizes are shown in Fig. 4 and agree quite well with the known sizes of the corresponding mRNA species (Fig. 4; 11).

Since the NV gene of IHNV has not been reported to exist in any other rhabdovirus, its position on the genome between the G and L gene was of great interest. Measurements of the pG480 and pNV58 R-loops showed that these sequences were very close and raised the possibility that they might overlap (Table 3). To obtain more accurate measurements, double R-loops of each of these plasmids with pM219, the most proximal gene, were carried out (Fig. 3, Table 4). The gap measurements between M2 and G and between M2 and NV hybridizations showed a better separation of the G and NV R-loops, indicating that they do not overlap but are adjacent. This is supported by the fact that the insert sequences of pG480 and pNV58 do not cross-hybridize. The cDNA insert of pNV58 is 420 base pairs in length (Table 1) and thus represents a minimum of 73% of the mRNA sequence (575 bases; molecular weight, 1.95×10^5; (11). If one excludes a 100-base polyadenylic acid tail from the mRNA length, then this insert could contain as much as 89% of the mRNA coding sequence. This explains the proximity of the R-loop measurements between the pNV58 insert and the pG480 insert from the adjacent G gene. The possibility that the NV and G gene sequences overlap

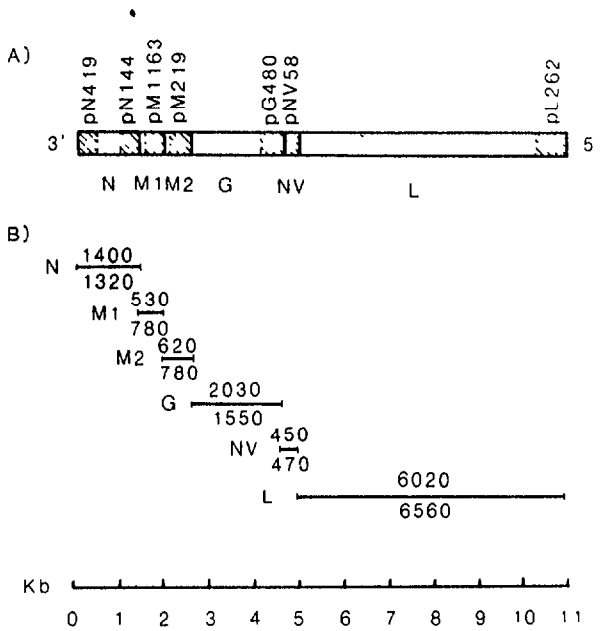

FIG. 4. Physical map of the IHNV genome. (A) Physical map showing the order of genes on the genome (delineated by heavy vertical lines) and locations of cloned sequences (shaded areas) as determined by R-loop measurements (Tables 3 and 4). (B) Horizontal lines represent individual genes. Numerical values (in nucleotides) above each line are gene sizes estimated from R-loop measurements, and values below each line are the corresponding mRNA sizes (11), excluding a polyadenylic acid tail of 100 residues. Kb, kilobases.

also is excluded by the mRNA blot hybridizations and hybrid selection results. If the NV and G genes overlapped, one would expect mRNA blot hybridization of pNV58 to both mRNA bands 2 and 5 and hybrid selection by pNV58 of mRNA for both the NV and G proteins. No such results were seen.

However, the types of data which prove that the NV and G genes do not overlap cannot be used to conclusively rule out the possibility that the NV sequence overlaps, or is contained within, the 3′ end of the L gene. Due to the size of the L gene (Fig. 4) one would not expect hybridization between cDNA sequences from its opposite ends. Hybridization to mRNA band 1 on blots was inconsistent and difficult to detect. The quantity of mRNA 1 in various mRNA preparations was extremely small and variable (11), and its large size (molecular weight, 2.26×10^6; 6.56 kilobases; 11) may have resulted in a lower efficiency of blotting transfer. In addition, the increased problem of degradation of such a large RNA was evident in the high background of lower-molecular-weight hybridization on blots probed with pL232 (Fig. 1f). This inconsistency of blot hybridizations to mRNA 1 and the failure of mRNA 1 to translate in vitro (11) mean that the lack of double hybridization on mRNA blots and double selection during hybrid selection may be due to technical difficulties specific to mRNA 1.

The most straight-forward genetic arrangement is that the NV gene is a separate gene, located between the G and L genes. However, although there is no evidence to indicate that the NV gene sequence does overlap the L sequence, this is an alternative genetic arrangement which has not been ruled out.

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MANUSCRIPT # 3

Transcription *in vitro* of Infectious Haematopoietic Necrosis Virus, a Fish Rhabdovirus

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Transcription *in vitro* of Infectious Haematopoietic Necrosis Virus, a Fish Rhabdovirus

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SUMMARY

Transcription *in vitro* by the RNA polymerase of infectious haematopoietic necrosis virus (IHNV), a salmonid rhabdovirus, was investigated using different reaction conditions to maximize RNA synthesis. The use of HEPES buffer rather than Tris buffer, and the addition of *S*-adenosyl-L-methionine to the reactions resulted in a sixfold increase in RNA synthetic activity to 6400 pmol UMP incorporated/mg viral protein/hour. The RNA transcripts produced in this system contained polyadenylated species which co-migrated with IHNV mRNA species 2, 3, 4 and 5 from IHNV-infected cells. The transcripts were shown to be functional mRNA species by their ability to direct the synthesis of viral proteins *in vitro*.

Infectious haematopoietic necrosis virus (IHNV) is a rhabdovirus which infects salmon and trout and causes great economic losses in fish hatcheries in western North America (Pilcher & Fryer, 1978). The virion protein structure is similar to that of rabies virus (Hill *et al.*, 1975; McAllister & Wagner, 1975) and IHNV also encodes one non-virion (NV) protein (Kurath & Leong, 1985). The genome of IHNV is single-stranded, negative sense RNA which is approximately 10900 nucleotides long (Hill *et al.*, 1975; Kurath & Leong, 1985) and encodes six mRNA species which are resolved into five bands by denaturing gel electrophoresis (Kurath & Leong, 1985). In order to characterize IHNV transcription further we examined the activity of the IHNV RNA polymerase *in vitro*. A system for transcription of IHNV *in vitro* has been described previously, in which the transcripts produced ranged from 9S to 17S in size and no discrete species were identified (McAllister & Wagner, 1977). We have attempted to maximize the viral polymerase activity by modifying the reaction mixture. RNA transcripts produced *in vitro* were characterized by denaturing gel electrophoresis and cell-free translation.

The IHNV used in this study was obtained and propagated on a chinook salmon embryo cell line (CHSE-214) as previously described (Kurath & Leong, 1985). IHNV was purified as described previously (Kurath & Leong, 1985) but omitting the discontinuous sucrose gradient. The activity of the viral RNA polymerase was assessed in 100 µl reactions containing 15 µl of purified virus (7 to 30 µg viral protein per reaction). The optimal reaction mixture (determined as described below) contained 400 mM-HEPES buffer pH 8·0, 0·05% Triton X-100, 30 mM-NH_4Cl, 5 mM-$MgCl_2$, 4 mM-dithiothreitol, 0·5 mM each of ATP, CTP and GTP, and 0·001 mM-UTP. The labelled precursor, [5,6-^{3}H]UTP, was included to a final specific activity of 86·7 Ci/mmol. The methyl donor, *S*-adenosyl-L-methionine (SAM), was included at 0·5 mM unless otherwise indicated. Tris–HCl-buffered reactions were carried out in 100 µl volumes with 15 µl purified IHNV using the conditions previously described (McAllister & Wagner, 1977). Reactions were incubated for 1 h at 18 °C unless otherwise indicated. The amount of RNA synthesized was determined by incorporation of [^{3}H]UMP into acid-precipitable material. Transcription reactions *in vitro* were scaled up and incubated for 3 h in order to obtain larger

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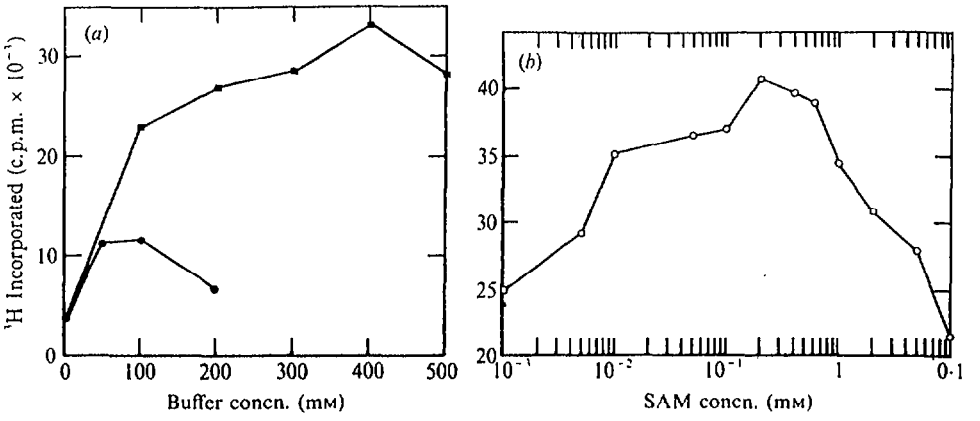

Fig. 1. (*a*) Effect of various concentrations of HEPES (■) or Tris (●) buffer on the synthesis of IHNV RNA *in vitro*. The rate of RNA synthesis was measured by the incorporation of [^{3}H]UMP into acid-precipitable material. (*b*) Stimulation of RNA synthesis *in vitro* by various concentrations of *S*-adenosyl-L-methionine.

quantities of the RNA reaction products for further analyses. Reactions were stopped by adding sodium dodecyl sulphate (SDS) to 0·5%; RNA was extracted with phenol and chloroform: isoamyl alcohol (24:1), and precipitated in ethanol. Precipitated RNA was pelleted, resuspended in water, and stored at −70 °C. Polyadenylated RNA was selected from the reaction products by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972).

Glyoxal treatment of RNA and subsequent electrophoresis on 1% agarose gels was carried out as described (McMaster & Carmichael, 1977) with the exception that the glyoxal (Fluka AG, Buchs, Switzerland) was more extensively deionized (Kurath & Leong, 1985). Fluorography was as described previously (Kurath & Leong, 1985). Polyadenylated RNA produced *in vitro* or in infected cells was translated in a cell-free, nuclease-treated rabbit reticulocyte lysate system (Bethesda Research Laboratories) as specified by the manufacturer. Reactions were carried out in 15 µl volumes for 1 h at 30 °C in the presence of [^{35}S]methionine (New England Nuclear, NEG-009A, 1166·5 Ci/mmol). Proteins were analysed by SDS–polyacrylamide gel electrophoresis on 10% polyacrylamide gels with a 4·75% stacking gel (Laemmli, 1970). Following electrophoresis at 20 mA, gels were fixed and fluorographed as described (Kurath & Leong, 1985).

A series of experiments were undertaken to define optimal conditions for RNA transcription *in vitro* by the RNA polymerase of IHNV. Sets of reactions were carried out in which only one reaction component was varied and the amount of tritiated UMP incorporated into TCA-precipitable material in 1 h was measured. In this way the concentration of each reaction component that stimulated the maximum amount of RNA synthesis was determined. Each set of reactions was carried out a minimum of three times.

An investigation of the buffer conditions showed that HEPES buffer stimulated threefold more RNA synthesis than Tris buffer (Fig. 1*a*). The optimum concentration of HEPES buffer was 400 mM, while the optimum for Tris buffer was 50 to 100 mM.

Table 1 summarizes the results from tests of several other reaction features. The pH of the HEPES buffer that allowed maximum RNA synthesis was 8·0. Detergent lysis of the virus particles was required for transcription, and maximum activity occurred in the presence of 0·05% Triton X-100. Three non-ionic detergents (Triton X-100, Triton N-101 and NP40) were tested and found to stimulate comparable levels of polymerase activity. Temperature studies revealed a fairly sharp optimum of activity at 16 to 18 °C. Tests of the requirement for a reducing agent showed that the optimal concentration of dithiothreitol was 4 mM, while reactions that included 2-mercaptoethanol showed significantly less activity. To define the monovalent cation requirements for the IHNV polymerase, KCl, NaCl, and NH_4Cl were included at

Table 1. *Optimal reaction conditions for IHNV transcription* in vitro

Reaction condition	Test range	Optimal value
Buffer	0–500 mM-HEPES 0–200 mM-Tris	400 mM-HEPES
pH	7·0–9·0	8·0
Detergent	0·0–0·10%	0·05% Triton X-100
Reducing agent	0–10 mM	4 mM-dithiothreitol
Temperature	0–30 °C	18 °C
Monovalent cation	0–100 mM	30 mM-NH_4Cl
Divalent cation	0–10 mM	5 mM-$MgCl_2$
SAM	0–10 mM	0·2–0·5 mM

concentrations ranging from 0 to 100 mM. Although all three stimulated significant activity, the presence of 30 mM-NH_4Cl in the reaction mixture consistently resulted in the highest enzyme activity. In the presence of 400 mM-HEPES the presence of a monovalent cation was not essential, and NH_4Cl concentrations greater than 60 mM were inhibitory. However, if the HEPES concentration was lowered to 100 mM the addition of NH_4Cl did stimulate enzyme activity significantly. Thus, the monovalent cation requirement could be at least partially fulfilled by high concentrations of HEPES buffer. The viral polymerase showed a definite requirement for a divalent cation, with a sharp optimum of activity at 5 mM-$MgCl_2$ (Table 1). RNA synthesis in the presence of manganese was at best 30% of the activity with magnesium. With the exception of the buffer and the temperature optima, these requirements of the IHNV polymerase are similar to those of rabies virus (Kawai, 1977), vesicular stomatitis virus (VSV) (Aaslestad *et al.*, 1971; Bishop, 1971; Moyer & Banerjee, 1975), and other fish rhabdoviruses (McAllister & Wagner, 1977; Roy *et al.*, 1975; Roy & Clewley, 1978). The temperature optimum agrees with the earlier report for IHNV polymerase activity (McAllister & Wagner, 1977), but the divalent cation studies differ in that manganese was not able to replace magnesium with equal efficiency.

The addition of the methylating agent SAM to the optimized transcription reactions resulted in a doubling of the amount of RNA synthesis. The minimum concentration of SAM capable of stimulating the viral polymerase was approximately 10 μM, and maximum activity was observed in the presence of 0·2 to 0·5 mM-SAM (Fig. 1*b*). The presence of SAM results in increased activity *in vitro* of the RNA polymerases of many viruses including the cyprinid rhabdovirus spring viraemia of carp virus (Roy & Clewley, 1978). Presumably, SAM acts as a methyl donor for capping of the mRNA 5′ end and could exert a stimulatory effect on transcription if required methylating compounds were limiting in the reaction.

The previous system described for IHNV transcription *in vitro* reported the activity of the polymerase to be 1040 pmol UMP incorporated/mg viral protein/h (McAllister & Wagner, 1977). Our IHNV preparations exhibited activities comparable to this when we used the Tris-buffered reaction conditions described by those authors. The polymerase activity obtained using our modified reaction conditions was 6400 pmol UMP incorporated/mg viral protein/h. This sixfold increase in activity is largely due to the use of HEPES buffer and the addition of SAM to the reactions. In comparison with the polymerases of other rhabdoviruses, this activity is higher than that reported for rabies virus (140 pmol GMP incorporated/mg viral protein/h) (Kawai, 1977), but still substantially lower than that of VSV (21 000 pmol UMP incorporated/mg viral protein/h) (Chang *et al.*, 1974; Moyer & Banerjee, 1975).

The kinetics of the RNA polymerase activity under the optimal conditions defined above was examined by determining the amount of tritiated UMP incorporated into RNA during various incubation times. The rate of RNA synthesis was linear for at least the first hour of incubation, and in some trials synthesis was linear for up to 3 h (data not shown).

RNA synthesized *in vitro* under the optimal conditions defined above was denatured by glyoxal treatment and examined by agarose gel electrophoresis (McMaster & Carmichael, 1977). The reaction products ranged in size from approximately $4{\cdot}0 \times 10^4$ to $5{\cdot}6 \times 10^5$ and contained three major discrete species which comigrated with IHNV mRNA species 3, 4 and 5 from

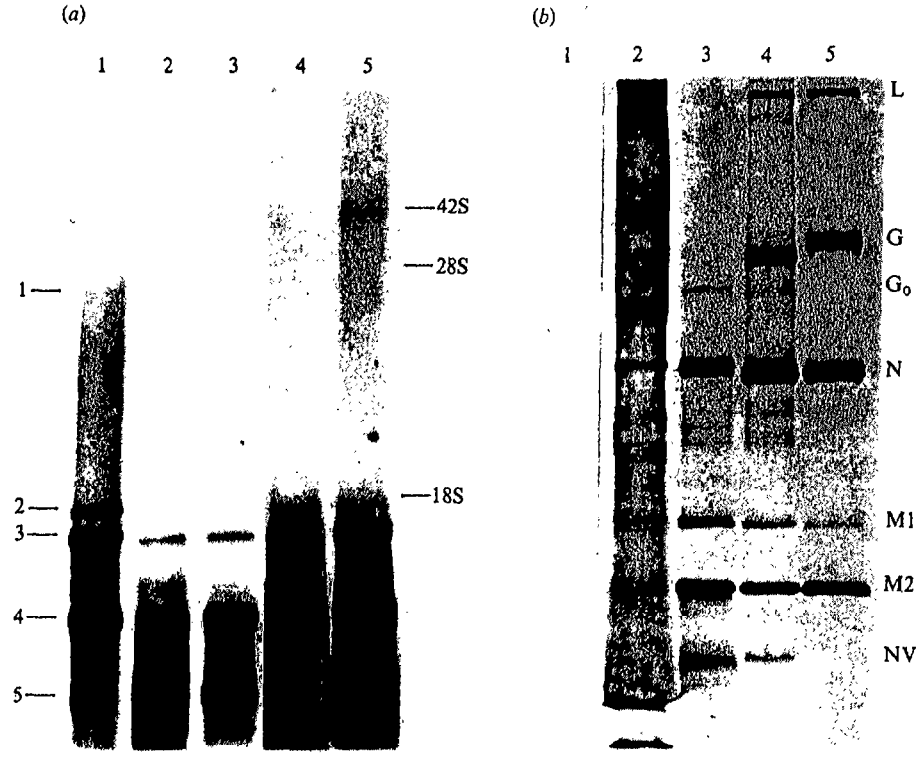

Fig. 2. (*a*) IHNV RNA was transcribed *in vitro* under the optimal conditions described in the text, denatured by glyoxal treatment (McMaster & Carmichael, 1977), and visualized by agarose gel electrophoresis and autoradiography. Distances of migration of RNA size markers are indicated on the right, and IHNV mRNA species are identified on the left. Lane 1, IHNV mRNA isolated from virus-infected CHSE-214 cells (Kurath & Leong, 1985); lane 2, unfractionated RNA from IHNV transcription reactions *in vitro*; lane 3, polyadenylated *in vitro* transcripts. Lanes 4 and 5 are darker exposures of lanes 2 and 3, which are included to show mRNA 2 in the *in vitro* transcripts. (*b*) Proteins translated from *in vitro* transcribed RNA in a cell-free rabbit reticulocyte system. Protein products were analysed by SDS–PAGE and autoradiography. Cell-free translation reactions were primed by the addition of: lane 1, no exogenous RNA; lane 2, polyadenylated RNA transcribed *in vitro*; lane 3, polyadenylated RNA from IHNV-infected CHSE-214 cells (Kurath & Leong, 1985). Lane 4 contains [^{35}S]methionine-labelled proteins from IHNV-infected cells, and lane 5 contains proteins from purified IHN virions (Kurath & Leong, 1985). IHNV proteins are identified on the right, G_0 is the unglycosylated form of the G protein.

IHNV-infected salmon cells (Fig. 2*a*, lane 2) (Kurath & Leong, 1985). There was also a small quantity of a species which comigrated with IHNV mRNA 2 (Fig. 2*a*, lane 4). IHNV mRNA 1, which encodes the large (L) protein, has a molecular weight of $2{\cdot}3 \times 10^6$ (Kurath & Leong, 1985). An RNA transcript of this size was never observed in reaction products *in vitro*, presumably because of its large size.

Fractionation of the RNA transcripts by chromatography on oligo(dT)-cellulose showed that 10 to 30% of the RNA was bound by the resin, indicating a significant proportion of molecules with polyadenylate tracts. Glyoxal gel electrophoresis showed that the polyadenylated RNA was mainly composed of the four distinct RNA species that comigrate with IHNV mRNA species 2, 3, 4 and 5 (Fig. 2*a*, lanes 3 and 5) (Kurath & Leong, 1985), and lacked most of the heterogeneous background material visible in unfractionated RNA. The non-polyadenylated RNA contained no discrete bands, and was presumed to be incomplete or degraded transcripts (not shown). The band of RNA migrating with the viral genome (Fig. 2*a*, lane 5) is most likely due to trapping of

labelled material by the large quantity of non-radioactive viral genome RNA in the preparation. This conclusion has been suggested by reconstitution experiments.

In order to determine whether the polyadenylated RNA transcribed *in vitro* was functional mRNA, it was used to prime a rabbit reticulocyte cell-free translation system. The [^{35}S]methionine labelled proteins synthesized *in vitro* are shown in Fig. 2(*b*). Unfortunately there was a background of heterogeneous material which may have been due to tritium-labelled RNA transcripts. Nevertheless, a protein comigrating with the viral nucleoprotein (N) was clearly visible, and there were faint bands that comigrated with the matrix proteins M1 and M2. The lack of visible glycoprotein (G) and NV protein products was not surprising since these proteins were translated less efficiently from IHNV mRNA prepared *in vitro* (compare lanes 2 and 3, Fig. 2*b*), and the absolute quantity of RNA obtained from the *in vitro* system is very low.

The sixfold increase in synthetic activity due to the modified reaction conditions and the detection of discrete, identifiable transcript species are both significant advances for this IHNV system relative to the system described previously (McAllister & Wagner, 1977). In addition, cell-free translation of the polyadenylated RNA transcripts to produce intact viral proteins shows that this transcription reaction is capable of producing functional viral mRNA *in vitro*. Therefore, this system is useful for both the production of viral nucleic acid and the study of the process of viral transcription in controlled conditions which are not influenced by the host cell.

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Nucleotide Sequence of a cDNA Clone Carrying the Glycoprotein Gene of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus

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Nucleotide Sequence of a cDNA Clone Carrying the Glycoprotein Gene of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus†

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The nucleotide sequence of the mRNA encoding the glycoprotein of infectious hematopoietic necrosis virus was determined from a cDNA clone containing the entire coding region. The G-protein cDNA is 1,609 nucleotides long (excluding the polyadenylic acid) and encodes a protein of 508 amino acids. The predicted amino acid sequence was compared with that of the glycoprotein of the Indiana and New Jersey serotypes of vesicular stomatitis virus and with the glycoprotein of rabies virus, using a computer program which determined optimal alignment. An amino acid identity of approximately 20% was found between infectious hematopoietic necrosis virus and the two vesicular stomatitis virus serotypes and between infectious hematopoietic necrosis virus and rabies virus. The positions and sizes of the signal sequence and transmembrane domain and the possible glycosylation sites were determined.

Infectious hematopoietic necrosis virus (IHNV) belongs to the family *Rhabdoviridae* and is a major viral pathogen of young salmon and trout. The mortality rate is high for infected fish, and death results from the destruction of the kidneys and spleen (1–3). The genome of the virus consists of an unsegmented single-stranded RNA of ca. 10.9 kilobases with negative polarity (24, 31). Although the virus is morphologically similar to vesicular stomatitis virus (VSV) and rabies virus (2, 15, 30), the viral genome is unique in encoding six, rather than five, viral proteins (24). These viral proteins include the virion polymerase (L), a surface glycoprotein (G), two matrix proteins (M1 and M2), a nucleocapsid protein (N), and a nonvirion protein (NV). These proteins have been mapped on the viral genome in the following order: 3′-N-M1-M2-G-NV-L-5′ (24).

The glycoproteins of rhabdoviruses are integral membrane proteins that are synthesized on the rough endoplasmic reticulum and vectorially transported through the smooth membrane and Golgi apparatus (44). In a manner similar to other transmembrane or secretory glycoproteins, they undergo signal peptide processing and core glycosylation during this transport (6). The viral glycoproteins of VSV and rabies, influenza, and respiratory syncytial viruses are characterized by the presence of an N-terminal hydrophobic signal sequence that is cleaved during transport and a C-terminal membrane anchorage domain (9, 36).

The IHNV glycoprotein, G, is a membrane-associated protein which forms spikelike projections on the surface of the mature virion (30). Antiglycoprotein serum neutralizes viral infectivity, and immunization with purified glycoprotein prevents subsequent lethal infection with IHNV (H. Engelking and J. Leong, unpublished data). Immunological studies with polyvalent antiglycoprotein sera have indicated that the glycoproteins are conserved among different geographic isolates of IHNV (18). Yet, these isolates exhibit differences in virulence, species specificity (M. Chen, Ph.D. thesis, Oregon State University, Corvallis, 1985), growth properties (33), and the apparent molecular weight of the G protein (17, 27, 28). To begin an investigation of the role of the glycoprotein in these events, we produced a cDNA clone of the mRNA for G protein and determined its nucleotide sequence. A comparison of the nucleotide and deduced amino acid sequences with other available sequences of VSV and rabies virus G genes revealed partial conservation of the sequence.

MATERIALS AND METHODS

Cells and virus. The chinook salmon embryo cell line (CHSE-214) used for propagating IHNV was provided by J. L. Fryer, Oregon State University, Corvallis. The cells were grown as monolayers in minimal essential medium (GIBCO Laboratories) supplemented with 5% fetal calf serum, 2 mM L-glutamine, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. The IHNV used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte Hatchery in Oregon. The fish cells were infected with IHNV at a multiplicity of infection of 0.001 and incubated at 15°C for 7 days. At that time the supernatant was harvested and centrifuged at 2,500 × *g* for 10 min at 4°C. The cell-free supernatant contained 0.5×10^8 to 1×10^8 50% tissue culture infective doses per ml. Virus purification was performed on sucrose gradients as described previously (28).

Synthesis and cloning of double-stranded cDNA. Polyadenylated intracellular RNA was prepared from CHSE-214 cells that were synchronously infected with IHNV in the presence of actinomycin D as described previously (24). The polyadenylated RNA was reverse transcribed to synthesize single-stranded cDNA in a standard reaction containing an oligo$(dT)_{12-18}$ primer (Collaborative Research, Inc.), placental RNase inhibitor (ENZO Biochemicals, Inc.), and reverse transcriptase (Life Science Div., The Mogul Corp.). Double-stranded cDNA was prepared according to the procedure of Gubler and Hoffman (13) in which the second strand of cDNA is primed by the RNA template in a replacement reaction including RNase H, *Escherichia coli* polymerase I, and *E. coli* DNA ligase (Bethesda Research Laboratories). The double-stranded cDNA was tailed with oligo(dC) residues, annealed to *Pst*I-

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cut oligo(dG)-tailed pUC8, and transformed into *E. coli* K-12 strain C600 SC181 (5).

Plasmid preparations, colony blots, and Southern blots. Plasmid DNAs were isolated on a large scale by the procedure of Holmes and Quigley (16). Small-scale plasmid preparations were done by a modification of the alkaline procedure (7) as described previously (23). Southern blots were carried out as described by Maniatis et al. (29) and Southern (39). Colony hybridizations were performed on Whatman 541 filter paper by the method of Taub and Thompson (42) and hybridized with a ^{32}P-labeled G-specific cDNA. The probe was made by nick translation of the 440-base pair insert of clone pG480, which contains sequences from the 3′ end of the coding sequence of the glycoprotein gene (23). Autoradiography was performed with Kodak X-AR5 films with Du Pont intensifying screens at −70°C.

Characterization of cDNA clones. Purified plasmids were cleaved with the restriction endonuclease *Pst*I (Bethesda Research Laboratories) to release the cloned insert DNAs. Plasmid and insert DNAs were separated on agarose and polyacrylamide gels, and the insert sizes were determined by comparison to a standard DNA marker (*Hin*dIII-restricted lambda DNA) after ethidium bromide staining. For the acrylamide gels the Laemmli gel system was used (26), but sodium dodecyl sulfate was omitted from all buffers. UV-illuminated gels were photographed with Polaroid type 47 film.

The *Pst*I inserts were separated from the plasmid vectors by electrophoresis on a low-melting-temperature agarose gel (Seakem; FMC Corp.) and purified by phenol-chloroform extraction. The isolated inserts were mapped by single and double restriction enzyme digestions. Electrophoresis of the resulting fragments was performed on polyacrylamide gels, and accurate sizing was done with *Hin*fI and *Hae*III pBR322 restriction fragment standards.

Determination of nucleotide sequences. A cDNA clone containing the entire coding sequence of the IHNV glycoprotein mRNA (pG8) was used for sequence analysis. A (G-NV) clone encoding the carboxy terminus of the glycoprotein gene and overlapping into the neighboring NV gene of IHNV (R. Gilmore and J. Leong, unpublished data) was used to confirm the sequence at the carboxy end of the glycoprotein gene. Suitable fragments were used to clone in both orientations into the polylinker sequences of the cloning vectors M13mp18 and M13mp19. The DNA sequence analysis was done by the dideoxynucleotide method of Sanger et al. (38). The entire sequence of the G8 insert was determind on both strands. In one series, fragments generated by the restriction endonucleases *Taq*I and *Pst*I were subcloned into M13mp18 and M13mp19. In the other series pG8 was digested with *Hin*dIII and random ends were generated with fast Bal31 nuclease (International Biotechnologies Inc.) by digesting for various times (1 to 5 min) under the conditions recommended by the manufacturer. The mixture was digested with *Bam*HI, and the resulting Bal31 deletion fragments were cloned into the *Bam*HI and *Sma*I sites of M13mp18. The nucleotide sequence data were analyzed for translational open reading frames, restriction endonuclease sites, and codon usage. Rabies virus, VSV, and IHNV hydropathicity profiles were determined by the program of Kyte and Doolittle (25).

RESULTS

Cloning of the cDNA of the glycoprotein gene. Poly(A)-containing RNA from IHNV-infected salmon cells was used to synthesize oligo(dT)-primed single-stranded cDNAs. The electrophoretic profile of the cDNAs consisted of prominent bands of ca. 7.0, 1.6, 1.4, 0.9, and 0.6 kilobases (Fig. 1), which corresponded in size to IHNV mRNA bands 1, 2, 3, 4, and 5, respectively (24). Double-stranded cDNA copies were prepared and cloned into the *Pst*I site of pUC8.

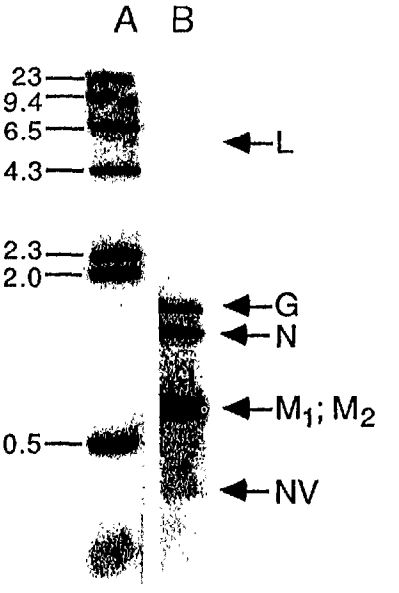

FIG. 1. Reverse transcription of IHNV mRNA. ^{32}P-labeled first-strand cDNA was prepared by reverse transcription and analyzed by electrophoresis on an alkaline agarose gel. (A) ^{32}P-labeled lambda DNA digested with *Hin*dIII was used as a marker. (B) Identities of the cDNA bands in the autoradiogram are indicated.

Twenty-five clones containing G-specific sequences were identified by colony blot hybridization to a ^{32}P-labeled probe from a clone of the carboxy terminus of the G gene as previously reported (23). Seven clones containing inserts ranging in size from 1,175 to 1,600 base pairs were isolated for further study. One of these large clones, pG8, was used for determination of the restriction map (Fig. 2) and nucleotide sequence of the entire G gene cDNA (Fig. 3). Three other clones with an insert size of ca. 1,600 base pairs were found to be identical to pG8 by single-base sequence analysis.

Sequencing strategy. Clone pG8 was sequenced according to the strategy shown in Fig. 2. The DNA sequence of the viral insert in pG8 is shown in Fig. 3 in the messenger sense. The recombinant contains 1,609 residues excluding 17 A's at the 3′ end representing the oligo(dT)-primed cDNA construction. The accuracy of the sequence was ascertained by A-track sequence analysis of several of the smaller clones. The precise delineation of the 3′ termini of the G gene was determined by sequence analysis of clones containing the intervening sequences between the G and NV genes.

Nucleotide sequence of IHNV G gene. There are two ATG start codons at positions 49 and 58 in the cDNA clone, both within a single open reading frame encoding a protein of 508 amino acids. The initial sequence of this single open reading frame from positions 46 to 61 is ACAATGGACACCATGA. Both ATG codons are preceded with A in the −3 position and the first ATG codon has a G at the +4 position after the

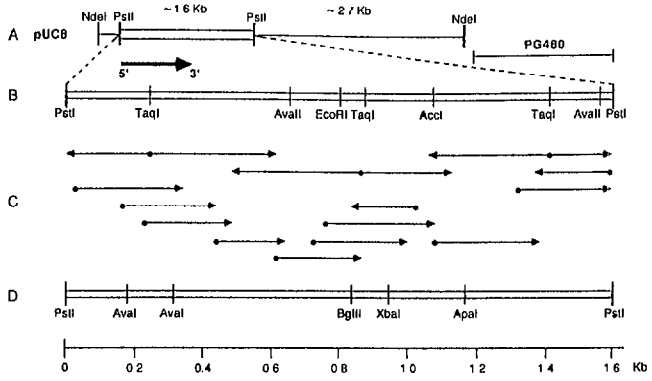

FIG. 2. Physical map and sequencing strategy for cDNA clone pG8 of the IHNV glycoprotein gene. Double lines represent cDNA and thin lines represent vector DNA. (A) The IHNV G cDNA is located between the *Pst*I sites of pUC8. The solid arrow indicates the orientation on the cDNA in pUC8. The cDNA clone pG480 is located at the 3′ end of the mRNA. (B, D) Restriction map and endonuclease cleavage sites used for DNA sequencing within the cloned fragment. (C) Sequencing strategy. Arrows indicate the regions sequenced and the direction of sequencing.

start of the ATG codon. The optimal sequence for initiation by eucaryotic ribosomes has been determined by a survey of the 5′ ends of eucaryotic mRNAs (20, 21) and defined by mutagenesis (22) to be ACCATGG. The purine in the −3 position occupies a dominant position for ribosome initiation and G in position +4 is also important. Thus, there is a "Kozak's box" at the first ATG start codon of the G gene open reading frame, and this suggests that the entire coding sequence for the G gene has been obtained.

The termination codon, TAA, at position 1573 occurs 34 nucleotides upstream of the poly(A) tail. A survey of the 50-base pair sequence preceding the polyadenylation site reveals no eucaryotic polyadenylation signal. This finding is similar to that reported for VSV (12, 35) in which the putative polyadenylation signal, ATTATAAAAAAA, is not homologous to the eucaryotic consensus sequence for polyadenylation.

Deduced amino acid sequence of G protein. The deduced protein of 56,795 daltons has an N-terminal hydrophobic signal peptide of 18 amino acids and a C-terminal hydrophobic anchorage domain of 27 amino acids (Fig. 3 and 4A). There is a signal peptidase recognition sequence, Ala-X-Ser, at position 18, with the cleavage site located after residue 20. There are five possible N-glycosylation sites (Asn-X-Ser/Thr) in the gene at amino acid positions 56, 400, 401, 438, and 506. In all probability, only one of the residues at 400 or 401 in the sequence Asn-Asn-Thr-Thr is used, and the amino acid residue at 506 is probably not used because it lies within the cytoplasmic domain of the glycoprotein.

DISCUSSION

The results in this paper show that the IHNV glycoprotein gene has many features characteristic of the membrane-associated glycoprotein gene of negative-stranded RNA viruses. The predicted translation product of the IHNV G gene has a domain of 20 amino acids at the N terminus which might act as a signal peptide. This includes a central core of hydrophobic amino acid residues in the form of three repeated pairs of Leu-Ile, which is unusual for signal peptides (34). This finding may reflect a difference in the membrane structure of fish and mammalian cells (32). The threonine residues at positions 3, 6, 7, and 15 are found at the beginning and near the end of the central core of the signal peptide, as has been reported for other signal peptides (34). Moreover, Ala at position 18 occupies a +4 position following the end of the central core sequence of Leu-Ile-Leu-Ile-Leu-Ile. The placement of Ala at this position is a frequent finding in other signal peptides.

The putative signal peptide cleavage site, Ala-Asn-Ser, at position 18 is consistent with possible signal peptidase cleavage sequences reported by Perlman and Halvorson (34). These authors examined presecretory signal peptides for 39 proteins from diverse procaryotic and eucaryotic sources. Their studies indicated that Ala-X-Ala, is the most frequently observed sequence preceding the signal peptide cleavage site. However, the last amino acid position in that triplet could also be occupied by Gly or Ser; thus, Ala-Asn-Ser is a permissible recognition sequence for the peptidase.

For most signal peptides, a beta turn in the protein structure is typically found between the hydrophobic core and the signal peptidase cleavage site. Upon close examination of the deduced amino acid sequence of the signal peptide carboxy terminus (Fig. 3 and 4), a predicted beta turn in the protein structure appears at Gly, residue 17, which occupies a +3 position from the end of the hydrophobic core (8). Thus, a careful analysis of the deduced sequence for the IHNV glycoprotein indicates that the amino terminus has the salient features of other known signal peptides.

The determination of the nucleotide sequence of the mRNA encoding the IHNV G protein made it possible for us to examine the extent of relatedness between the predicted amino acid sequences of IHNV, VSV New Jersey and Indiana, and rabies virus. All four virus G proteins are approximately the same size, with 508, 517, 511, and 524 amino acids, respectively (4, 12, 37). The IHNV G protein was compared with each of the other viral glycoproteins in two-way alignments by using a computer program which provides an optimal alignment and a statistical significance for the match (10). The IHNV and VSV New Jersey or Indiana sequences have 93 (18.4%) and 108 (21.3%) identities, respectively, in the optimum alignment. A comparison of IHNV and rabies virus glycoprotein sequences revealed 95 (18.8%) identities. A resemblance score based on an arbitrary scoring system was computed for each comparison. Values of >80.0 indicate significant resemblance well above what could be expected by chance (11). For the IHNV-VSV New Jersey, IHNV-VSV Indiana, and IHNV-rabies virus comparisons, the resemblance scores were 82.3, 82.7, and 81.8, respectively (Table 1). These scores indicate that there is a similar evolutionary distance between IHNV and rabies virus and for IHNV and VSV.

There are regions within the IHNV G protein which show significantly greater homology with the other rhabdovirus G proteins than is indicated by the overall resemblance scores. A sequence of 16 amino acid residues at positions 310 to 327 in IHNV and 15 amino acids at positions 290 to 315 in rabies virus exhibits an amino acid homology of 62.5% (Fig. 5). A different region of homology of >50% was found between VSV New Jersey and IHNV glycoproteins at positions 170 to 190 (11 of 20, 55%) and for VSV Indiana and IHNV at positions 171 to 190 (12 of 19, 63%) on the IHNV sequence (Fig. 5). None of these regions corresponds in position to the highly homologous region shared between rabies virus and

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                                                                1                                                  10
                                                              met asp thr met ile thr thr pro leu ile leu ile leu ile thr cys gly
TCTTTGTGCTTTGAGACCGAACGCAACTCGCAGAGACCCACCAAAACA              ATG GAC ACC ATG ATC ACC ACT CCG CTC ATT CTC ATT CTA ATC ACC TGC GGA    99

        20                                                 30                                                  40
ala asn ser gln thr val lys pro asp thr ala ser glu ser asp gln pro thr trp ser asn pro leu phe thr tyr pro glu gly cys
GCA AAC AGC CAA ACC GTC AAA CCC GAC ACC GCA AGC GAA TCA GAC CAA CCC ACC TGG TCA AAC CCG CTC TTC ACC TAT CCC GAG GGA TGC   189

        50                                                 60                                                  70
thr leu asp lys leu ser lys val asn ala ser gln leu arg cys pro arg ile phe asp asp glu asn arg gly leu ile ala tyr pro
ACT CTG GAC AAG CTC TCT AAG GTC AAT GCC TCT CAA CTG AGA TGC CCA AGG ATC TTC GAT GAT GAG AAC AGG GGG CTA ATT GCC TAT CCC   279

        80                                                 90                                                 100
thr ser ile arg ser leu ser val gly asn asp leu gly asp ile his thr gln gly asn his ile his lys val leu tyr arg thr ile
ACA TCC ATC CGG TCT CTG TCA GTC GGA AAC GAC CTC GGG GAC ATT CAC ACC CAA GGG AAC CAC ATC CAC AAA GTC CTG TAC CGC ACC ATC   369

        110                                                120                                                130
cys ser thr gly phe phe gly gly gln thr ile glu lys ala leu val glu met lys leu ser thr lys glu ala gly ala tyr asp thr
TGC TCA ACA GGG TTC TTC GGG GGT CAG ACG ATA GAG AAG GCG CTT GTA GAA ATG AAA CTC TCT ACG AAA GAA GCA GGG GCG TAT GAC ACC   459

        140                                                150                                                160
thr thr ala ala ala leu tyr phe pro ala pro arg cys gln trp tyr thr asp asn val gln asn asp leu ile phe tyr tyr thr thr
ACA ACC GCA GCC GCT CTG TAC TTC CCA GCT CCC CGA TGC CAA TGG TAC ACT GAC AAC GTA CAA AAT GAT CTC ATC TTC TAC TAC ACA ACC   549

        170                                                180                                                190
gln lys ser val leu arg asp pro tyr thr arg asp phe leu asp ser asp phe ile gly gly lys cys thr lys ser pro cys gln thr
CAA AAG AGT GTT CTG AGA GAT CCC TAC ACC AGA GAC TTT CTG GAC TCA GAT TTT ATT GGA GGA AAA TGC ACC AAA TCA CCC TGC CAG ACT   639

        200                                                210                                                220
his trp ser asn val val trp met gly asp ala gly ile pro ala cys asp ser ser gln glu ile lys ala his leu phe val asp lys
CAT TGG TCC AAC GTA GTT TGG ATG GGT GAT GCA GGG ATA CCA GCC TGT GAT TCC AGC CAA GAG ATA AAA GCT CAC CTC TTT GTT GAT AAA   729

        230                                                240                                                250
ile ser asn arg val val lys ala thr ser tyr gly his his pro trp gly leu his arg ala cys met ile glu phe cys gly lys gln
ATC TCC AAT CGG GTC GTG AAG GCA ACG AGC TAC GGA CAC CAC CCT TGG GGA CTG CAT CGG GCC TGT ATG ATT GAA TTC TGT GGG AAA CAG   819

        260                                                270                                                280
trp ile arg thr asp leu gly asp leu ile ser val glu tyr asn ser gly ala glu ile leu ser phe pro lys cys glu asp lys thr
TGG ATA CGG ACA GAT CTC GGT GAC CTG ATA TCT GTC GAA TAC AAT TCT GGA GCA GAA ATC CTC TCG TTC CCG AAG TGT GAG GAC AAG ACG   909

        290                                                300                                                310
met gly met arg gly asn leu asp asp phe ala tyr leu asp asp leu val lys ala ser glu ser arg glu glu cys leu glu ala his
ATG GGG ATG AGG GGA AAC TTG GAT GAC TTT GCC TAT CTA GAC GAT CTG GTG AAG GCC TCT GAG AGC AGA GAG GAA TGT CTT GAG GCG CAC   999

        320                                                330                                                340
ala glu ile ile ser thr asn ser val thr pro tyr leu leu ser lys phe arg ser pro his pro gly ile asn asp val tyr ala met
GCC GAG ATA ATA TCA ACA AAC AGT GTG ACT CCA TAC CTC CTA TCC AAG TTC CGA TCT CCA CAT CCC GGA ATA AAT GAC GTC TAC GCT ATG  1089

        350                                                360                                                370
his lys gly ser ile tyr his gly met cys met thr val ala val asp glu val ser lys asp arg thr thr tyr arg ala his arg ala
CAC AAA GGC TCC ATC TAT CAC GGG ATG TGC ATG ACG GTC GCT GTG GAC GAG GTA TCC AAG GAC AGG ACG ACG TAC AGG GCC CAT CGC GCT  1179

        380                                                390                                                400
thr ser phe thr lys trp glu arg pro phe gly asp glu trp glu gly phe his gly leu his gly asn asn thr thr ile ile pro asp
ACC AGC TTC ACG AAA TGG GAA CGA CCC TTT GGG GAT GAG TGG GAG GGC TTT CAC GGA TTG CAC GGA AAC AAC ACC ACC ATT ATT CCA GAC  1269

        410                                                420                                                430
leu glu lys tyr val ala gln tyr lys thr ser met met glu pro met ser ile lys ser val pro his pro ser ile leu ala phe tyr
CTG GAG AAA TAC GTC GCC CAG TAC AAG ACG AGC ATG ATG GAA CCG ATG AGC ATC AAA TCC GTA CCC CAT CCA AGC ATC CTG GCC TTC TAC  1359

        440                                                450                                                460
asn glu thr asp leu ser gly ile ser ile arg lys leu asp ser phe asp leu gln ser leu his trp ser phe trp pro thr ile ser
AAT GAG ACA GAC TTA TCA GGG ATC TCC ATC AGG AAA TTG GAC TCA TTC GAC CTT CAA TCA CTC CAC TGG AGT TTC TGG CCC ACA ATC TCC  1449

        470                                                480                                                490
ala leu gly gly ile pro leu val leu leu leu ala val ala ala cys cys cys trp ser gly arg pro pro thr pro ser ala pro gln
GCA CTG GGT GGG ATT CCC CTT GTT CTC CTC CTT GCT GTT GCC GCG TGC TGC TGC TGG TCA GGG AGA CCT CCC ACT CCC TCC GCG CCG CAG  1539

        500                                508
ser ile pro met tyr his leu ala asn arg ser OC
AGT ATC CCC ATG TAT CAC CTG GCA AAC CGG TCC TAA AGGACTCAATCTTCACTTCCTCCCCACCAGACAG                                      1609
```

Translated Mol. Weight = 56794.71

Codon Usage:

6/UUU/phe	8/UCU/ser	6/UAU/tyr	5/UGU/cys
13/UUC/phe	12/UCC/ser	14/UAC/tyr	11/UGC/cys
1/UUA/leu	11/UCA/ser	1/UAA/OC	0/UGA/OP
3/UUG/leu	1/UCG/ser	0/UAG/AM	11/UGG/trp
5/CUU/leu	2/CCU/pro	5/CAU/his	0/CGU/arg
14/CUC/leu	15/CCC/pro	13/CAC/his	2/CGC/arg
4/CUA/leu	7/CCA/pro	9/CAA/gln	3/CGA/arg
14/CUG/leu	5/CCG/pro	5/CAG/gln	5/CGG/arg
9/AUU/ile	6/ACU/thr	6/AAU/asn	4/AGU/ser
16/AUC/ile	17/ACC/thr	12/AAC/asn	9/AGC/ser
8/AUA/ile	8/ACA/thr	14/AAA/lys	5/AGA/arg
14/AUG/met	9/ACG/thr	11/AAG/lys	6/AGG/arg
5/GUU/val	7/GCU/ala	12/GAU/asp	4/GGU/gly
9/GUC/val	12/GCC/ala	21/GAC/asp	2/GGC/gly
5/GUA/val	9/GCA/ala	9/GAA/glu	12/GGA/gly
4/GUG/val	5/GCG/ala	14/GAG/glu	14/GGG/gly

FIG. 3. Nucleotide sequence of the IHNV G mRNA, the predicted glycoprotein sequence, and the codon usage.

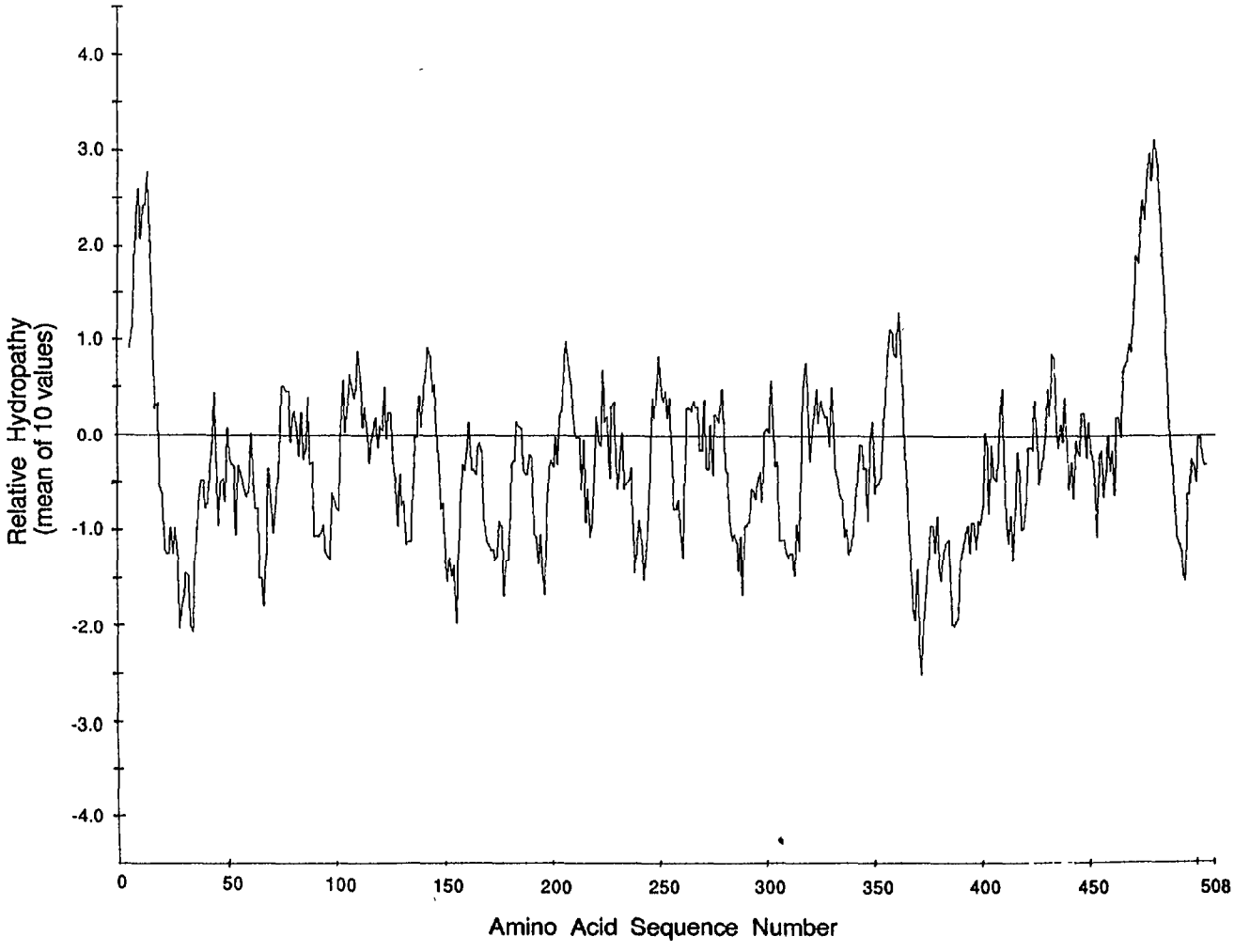


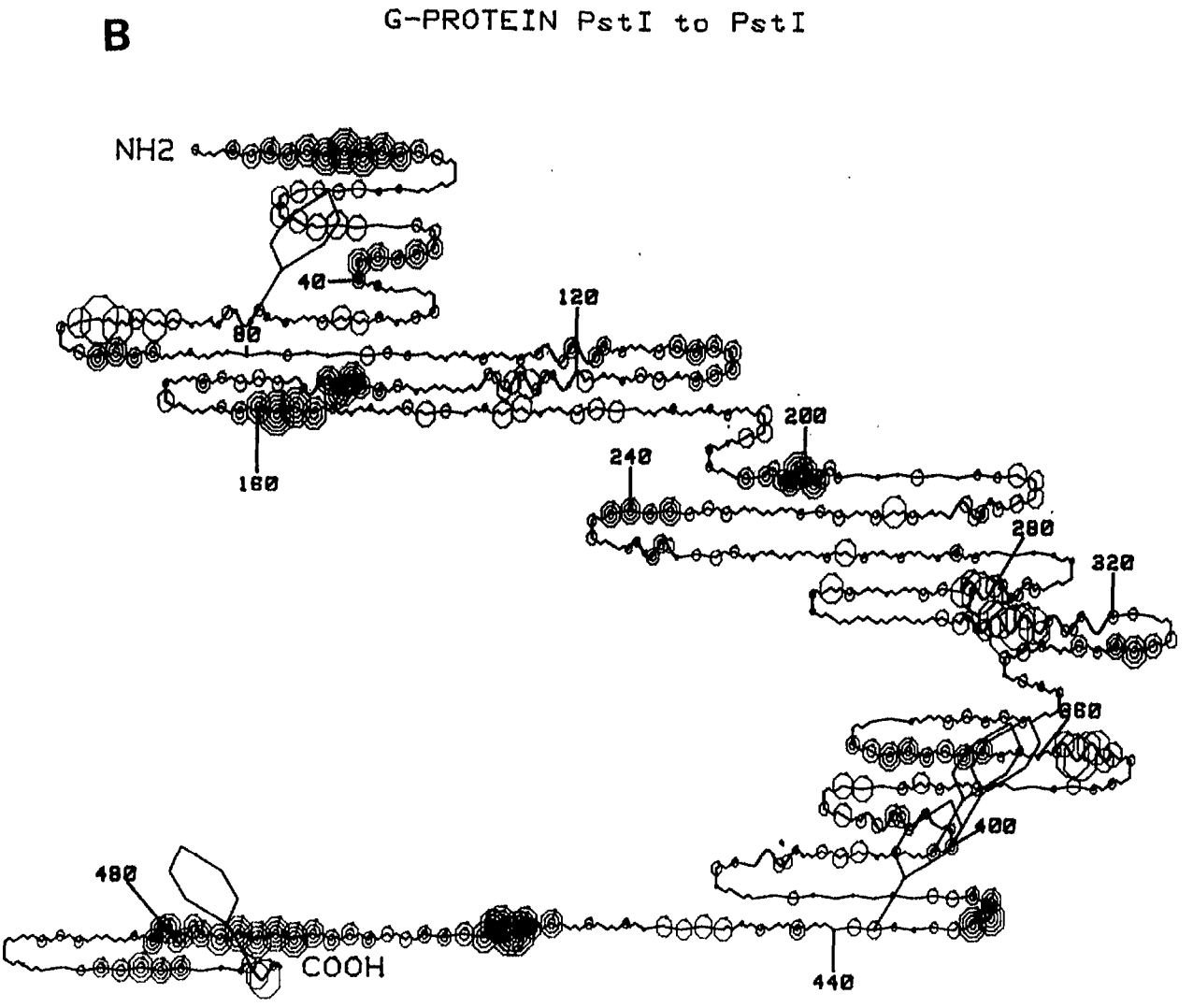
FIG. 4. Hydropathy plot and secondary structure of the IHNV glycoprotein. (A) Hydrophobicity is shown by positive values. Relative hydropathy values reflect a mean of 10 consecutive amino acid residues by the method of Kyte and Doolittle (25) and were plotted against the amino acid sequence number. (B) Predicted secondary structure and hydropathy of the IHNV glycoprotein gene. Open circles indicate hydrophilic regions and shaded circles indicate hydrophobic areas. The radius of a circle over a residue position is proportional to the average hydrophilicity or hydrophobicity as calculated for that residue and the next five residues. Numbers correspond to amino acid residues from the NH_2-terminal methionine. The black open hexagonal structures point to predicted glycosylation sites at asparagine residues 56, 400, 401, 438, and 506. Rapid zig-zag lines indicate β sheets with α-carbons alternating above and below the chain. Random coils are represented as gently undulating straight lines and α-helices are shown as sine waves. This model of the IHNV glycoprotein was generated by E. Golub, University of Pennsylvania (43).

VSV near the carboxy terminus that was identified by Rose et al. (36).

Cysteine and proline residues are often highly conserved amino acids. Thus, the similar positioning of the cysteine residues in all four proteins is notable. Of the 16 cysteine residues in IHNV G protein, 9 were aligned with 9 of the 17 cysteine residues in rabies virus G protein. For VSV Indiana, 12 of the 15 cysteine residues are aligned with IHNV cysteine residues (Fig. 5 and 6). Similarly, 6 of 29 proline residues in IHNV G protein were aligned with 6 of 27 proline residues in the VSV G protein. For rabies virus, 10 of 31 proline residues in its G protein were aligned with the proline of IHNV G protein. A three-way comparison of the VSV Indiana, IHNV, and rabies virus glycoprotein sequences indicates that seven of the cysteine residues can be aligned among all three proteins (Fig. 6).

Following the algorithm of Kyte and Doolittle (25), we established the hydropathy profile for the IHNV glycoprotein (Fig. 4A) and compared it with VSV and rabies virus. All four proteins have hydrophobic signal peptides at their amino termini and hydrophobic transmembrane segments at the carboxy termini. There were no striking amino acid sequence homologies in any of these corresponding domains. Yet, the positions of corresponding cysteine and proline residues are maintained (Fig. 5 and 6). These similarities point to a close evolutionary relationship among these rhabdoviruses.

A computer prediction of the secondary structure of the G-gene product is shown in Fig. 4B and is based on the Chou and Fasman calculations (8) for protein structure. Near the carboxy terminus of the IHNV G protein is a very long region of the polypeptide chain that is free of beta turns. The length of this region (54 amino acid residues) is striking because the same region in rabies virus is only 28 residues in



length (43). This region encompasses the transmembrane domain, and the difference in fish and mammalian cell membranes appears to be reflected in these glycoproteins.

The overall base content of the IHNV G gene is 52 mol% guanine plus cytosine (G+C). This base content is somewhat higher than the 40 to 46 mol% G+C for the VSV glycoprotein-coding loci and the 48 mol% G+C for the rabies virus glycoprotein gene. The codon usage for IHNV G protein is given in Fig. 3. Several interesting features emerge from these data. First, there is a strong bias towards codons ending in C or G, representing 62% of the 508 IHNV G-protein codons. For the homologous gene in VSV, only 43% of the codons end in C or G. In contrast to this, there is a bias against codons having the dinucleotide CG in the first and second or second and third positions. For IHNV, on a random basis one would predict this dinucleotide to occur 104 times within the G-coding locus, whereas only 30 are observed. On the other hand, if the frequency of codons containing C in the third position and G in the first position is considered (i.e., between adjacent codons), random assortment would predict 37 CG pairs, and 34 such dinucleotides are found. In the VSV genes encoding the G, M, N, and NS proteins, and for the rabies virus G protein, there is an overall deficiency of the CG dinucleotide including adjacent codon positions and noncoding nucleotides (37).

Because vertebrate genomes are known to be deficient in CG (41), it is possible that this dinucleotide frequency is kept low since the cytosine in the first position is a target for methylation and, consequently, could be a potential site for mutation (14). This rationale may not be applicable to the rhabdoviruses since infection probably does not occur via a

TABLE 1. Comparison of G-protein homology[a]

Virus	% Homology (no. of identical amino acids)		
	VSV-G	RV-G	IHNV-G
VSV-G	X	20.6% (105)	21.3% (108)
RV-G	84.4	X	18.8% (95)
IHNV-G	82.7	81.8	X

[a] The numerical resemblance score for the respective virus pairs was computed by R. Doolittle (11). VSV-G, Glycoprotein gene of VSV, Indiana strain; RV-G, glycoprotein gene of rabies virus, ERA strain; IHNV-G, glycoprotein gene of IHNV, Round Butte strain; X, identity.

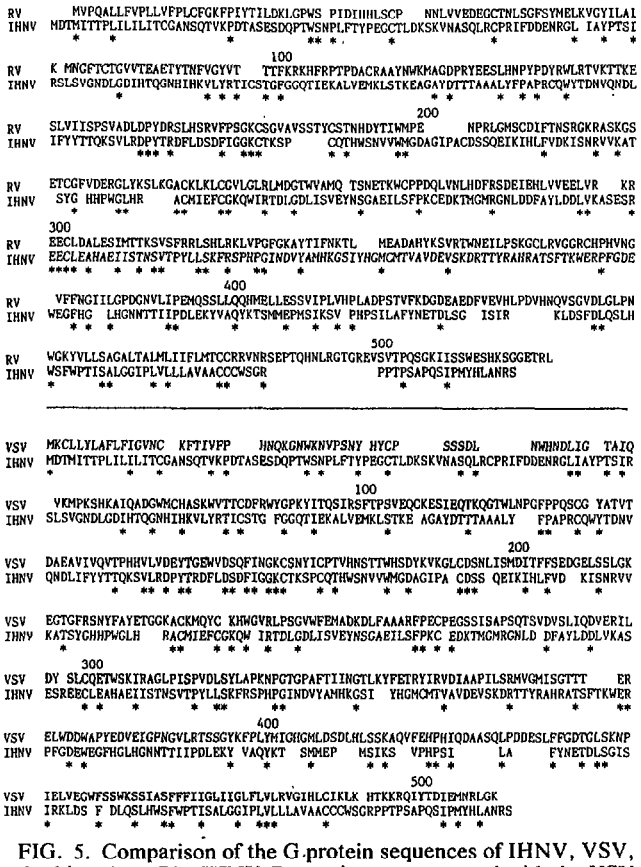


FIG. 5. Comparison of the G protein sequences of IHNV, VSV, and rabies virus. The IHNV G protein was compared with the VSV and rabies virus G proteins in two-way alignments by the method of Doolittle (10).

DNA intermediate. However, it may reflect a viral adaptation to utilize the isoaccepting tRNAs present in the host cell.

Asn-X-Thr/Ser sequences are known to be possible sites for glycosylation of eucaryotic polypeptides (19, 40). Oligosaccharide side chains may help to expose certain antigenic epitopes or receptor recognition sites to the surface of the molecule and are, therefore, biologically important sites in the infection cycle of the virus. It would seem reasonable that these sites would be conserved. When these possible N-glycosylation sites on the IHNV G protein were compared with those sites on either rabies virus or VSV, the glycosylation site at position 56 was identified as one shared with the rabies virus G protein at the corresponding position.

The presence of a very hydrophilic domain extending from amino acid 365 to 450 which is not evident in either VSV or rabies virus was also noted. Within this region are two potential glycosylation sites, the first at position 400 or 401 (these are overlapping sites) and the second at position 438. It is not known if any or all of these sites are glycosylated in the mature, native protein. However, this region is unique to IHNV, is exposed at the outer membrane, and thus may represent an important antigenic site. Because immunity against IHNV is elicited by the viral glycoprotein (Engelking and Leong, unpublished data), this unique hydrophilic region may be a candidate for further study in the production of a subunit vaccine against IHNV infection.

Comparisons of structural features such as overall amino acid homology, potential glycosylation sites, the cysteine

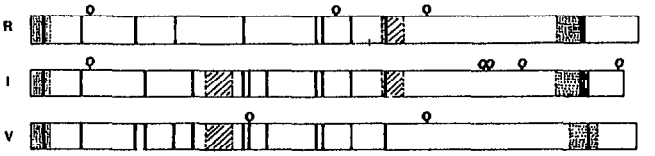


FIG. 6. Schematic representation of a three-way comparison of the G protein sequences of IHNV, VSV, and rabies virus. The N terminus is located on the left side. The stippled areas represent the signal peptide at the N terminus and the transmembrane domain at the C terminus. The hatched areas represent the homologous region between IHNV and VSV and the homologous region between IHNV and rabies virus. Potential glycosylation sites are indicated by open circles. The solid vertical lines represent matching cysteine residues. Only those cysteine residues matched in at least two of the G proteins have been included in this representation.

positions, transmembrane regions, or the signal peptide domain between IHNV and other rhabdovirus G proteins provide strong evidence for relatedness in the three-dimensional structure of these proteins. Future studies on the processing and transport of the IHNV G protein in fish cells may enable us to compare these processes in these diverse species.

ACKNOWLEDGMENTS

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15

Vaccination against Infectious Haematopoietic Necrosis*

J. C. Leong, J. L. Fryer and J. R. Winton

THE DISEASE PROBLEM

Economic significance – an overview

Infectious haematopoietic necrosis is a disease of certain salmonid fish native to the North Pacific Rim. The disease was first reported in 1953 as the cause of mortality among sockeye salmon (*Oncorhynchus nerka*) in the state of Washington.[1] Since that time, infectious haematopoietic necrosis virus (IHNV) has been recovered from susceptible salmonid species from Alaska to Northern California[2,3] and numerous epizootics have occurred among hatchery reared fish within this range. In 1972, the virus was recovered from sockeye salmon in Hokkaido, Japan.[4] IHNV has also been found in rainbow trout (*Salmo gairdneri*) and chum salmon (*Oncorhynchus keta*) on Honshu where it has spread widely. More recently the virus has been recovered from stocks of rainbow trout in Taiwan.[5] The authors are unaware of any reports of isolations of IHNV from the Soviet Far East or from Korea. While the most severe IHNV epizootics have been observed in hatcheries, natural outbreaks of IHNV have also been reported. The virus was isolated from young sockeye salmon at Chilko Lake, British Columbia, where 10–20 per cent of the salmon fry in the lake died of IHN.[6]

Since its discovery, the incidence of IHNV has increased dramatically, and the only available control measure for the disease has been destruction of the infected fish population. Because of the economic impact of IHNV on stocks of salmon and trout, development of vaccines and appropriate delivery systems have become important areas for research.

Populations at risk

IHNV produces a severe disease among fry and juvenile fish. Approximately 4 days after exposure to the virus, the infected fish become darker and show

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haemorrhages at the bases of fins. In the advanced stages of the disease, petechial haemorrhages appear in the musculature and in the mesenteries of the internal organs. Pathological examination of the infected fish indicates that the kidneys and spleen are the major target organs.

An epizootic of IHN is usually characterized by an abrupt onset and high mortality among the infected population. In fish up to 2 months of age, the mortality often exceeds 90 per cent. As fish become older susceptibility to IHNV decreases. Mortality is unusual in fish 2 years of age or older.

While virus may replicate *in vitro* between 5–20°C, most epizootics occur in the range of 10–14°C. A temperature sensitive strain has been recovered from fish at one location.[7] IHN has been controlled in fish at this site by raising the water temperature to 18°C; however, the number of carriers within the population has remained high.[8]

Transmission of IHNV

The route of transmission of IHNV in nature is uncertain. However, experimental studies have shown that the virus can be transmitted both horizontally and vertically. Horizontal water-borne transmission of IHNV has been demonstrated by a number of investigators.[9-11] In these studies, susceptible fish held in the effluent water from fish infected with IHNV were subsequently infected with IHNV. The initial sites of infection were the gills. The infection spread to visceral target organs such as the anterior kidneys and spleen. Infection by the oral route has been demonstrated by feeding the carcasses of infected fry to susceptible fish.[9,12]

The strongest evidence for vertical transmission comes from the association made between the appearance of the disease and the shipment of eggs from infected adults into geographical areas where the virus was not known to occur.[13-15] Mulcahy and Pascho[16] recently demonstrated IHNV transmission in naturally infected eggs and fry which were raised in virus-free water. However, Groberg[17] reported that when fertilized eggs were treated with iodophore and incubated to hatching in virus-free water, no virus was isolated nor signs of disease observed.

Carriers of IHNV

The existence of an IHNV carrier state is a contested issue among many scientists. In 1975, Amend[9] reported that in rainbow trout surviving an IHNV epizootic, the virus entered a latent state and was not recoverable until these fish reached sexual maturity. The virus was then found in milt or ovarian fluids. Other investigators have also attempted to detect a carrier

state by other methods with little success (Groberg, pers. comm.; Mulcahy, pers. comm.).[18] The ability to recover IHNV from fish surviving an epizootic only at the time of sexual maturity, raises the question as to whether the sexually mature fish expressed a latent virus or if viral reinfection occurred from some other reservoir.

If fish are reinfected with IHNV at spawning time, other reservoir(s) must be identified. The virus has been found to be extremely stable in sediments which may serve as sources of contamination (Mulcahy, pers. comm.). Other possible hosts, including resident wild fish and fish parasites, have been examined. Since the nature of the carrier state and the potential reservoirs of IHNV are essential elements in determining how and when a vaccine should be applied, they are important areas for research.

Pathogen heterogeneity

A serological comparison of rainbow trout, sockeye and chinook salmon isolates of IHNV using polyvalent rabbit antisera to whole virus indicated that while all isolates were similar, the chinook strain appeared different.[19] More recently, monoclonal antibodies (Mab) with neutralizing activity were used to distinguish among different virus strains.[20] At least three serological strains of IHNV were identified in this way.

Another method used for identifying strain differences among virus isolates was based on the differences in the migration patterns of the viral proteins on sodium dodecyl sulphate polyacrylamide gels (SDS–PAGE).[21] The virion protein patterns of 71 isolates of IHNV were analysed by SDS–PAGE of ^{35}S-methionine labelled intracellular viral proteins. With the molecular weights of the viral glycoproteins and nucleoproteins as a basis, five groups were identified. This method provided a reproducible and rapid way to identify virus isolates. In addition, the method permitted investigators to determine that a particular virus type may be endemic to an area and that different salmonid species within the area carry the same virus type.

Possible chemotherapeutic measures

At the present time there is no licensed chemotherapeutic drug for the control of IHNV infections in fish. However, research has been conducted in Japan to identify antiviral drugs for treatment of IHNV-infected fish. In a survey of 24 possible antiviral drugs, Hasobe and Saneyoshi[22] identified four compounds with *in vitro* anti-IHNV activity. These compounds were tested *in vivo* in IHNV infected steelhead fry. Only two compounds, 6-thioinosine and 5-hydroxyuridine, produced a modest reduction in mortality.

IHNV VACCINE

Types of vaccines

There are three types of vaccines that have been considered for IHNV control: attenuated, killed, and subunit viral vaccines produced by recombinant DNA technology. All three have had some success in inducing protective immunity in fish.

Among the selected criteria that an IHNV vaccine must meet, safety is the most important. A vaccine used in a system with an untreated outflow must be completely harmless for all potentially affected species in the watershed. Thus, an attenuated vaccine must be avirulent for resident fish and aquatic organisms. Also, the reversion frequency to virulence for a live vaccine must be carefully determined.

An ideal IHNV vaccine must also demonstrate effectiveness. Because fish are susceptible to lethal infection as early as newly-hatched fry and as late as yearlings, the vaccine should provide protective immunity during this period. It is a serious concern that the fish are most susceptible at a stage in their development when the immune system may be incapable of responding to antigenic stimulation. Leong[23] has shown that rainbow trout as small as 0.4 g are capable of mounting a protective immune response to IHNV. However, the duration of immunity to IHNV induced by the various types of vaccines has not been adequately ascertained.

Other important considerations in the development of an IHNV vaccine are those of cost and ease of administration. An ideal IHNV vaccine should be inexpensive to prepare and have a stable shelf-life. The vaccine must be easy to administer to large numbers of fish. It must provide protective immunity against all strains of IHNV and in all susceptible host species.

Vaccine trials

Laboratory trials have been conducted with the attenuated, killed and subunit IHNV vaccines. These studies have provided encouraging results which suggest that vaccination of salmonid fishes against the virus is possible. No field trial data have been reported. However, large-scale tests of vaccines conducted by private and public agencies are known to have yielded mixed results. In the laboratory, an attenuated IHN vaccine derived from a rainbow trout isolate was developed at Oregon State University. This vaccine provided protection of kokanee salmon against water-borne or intraperitoneal challenge with wild type virus (Table 15.1). Protection was found to last for at least 110 days.[24] Additional studies[25] showed chinook salmon could also be protected with the same vaccine. When rainbow trout were vaccinated, however, significant residual virulence was detected.[26]

Table 15.1 *Immunization of sockeye salmon with an attenuated vaccine against IHNV*[24]

Treatment	Challenge	Per cent mortality
Vaccinated	Injected	5
	Water-borne	5
Nonimmunized controls	Injected	100
	Water-borne	90

Sockeye salmon (0.9 g) were immunized by exposure to 6200 pfu/ml of water containing the attenuated vaccine for 48 h. After 25 days, fish were challenged by intraperitoneal injection of 40 LD_{50} or by exposure in water to 4×10^3 pfu/ml wild type IHN virus.

Further attenuation and testing of the virus was suspended due to the lack of commercial interest.

Two groups have reported success with vaccination of rainbow trout using killed preparations of IHNV. Amend[27] used β-propiolactone to inactivate IHNV and immunized trout fry by intraperitoneal injection. He found the immunized fish were resistant to a lethal challenge dose of IHNV (Table 15.2). More recently, Nishimura *et al.*[28] tested several methods of formalin inactivation of IHNV to determine the optimum inactivation schedule. Several of the formalin-killed vaccines were used to protect juvenile trout against lethal challenge. The vaccine was most effective when delivered by injection; however, hyperosmotic immersion was capable of stimulating limited immunity (Table 15.3).

Table 15.2 *Immunization of rainbow trout with a β-propiolactone-killed vaccine against IHNV*[27]

Treatment	Challenge	Per cent mortality
Vaccinated	Injected	4
Nonimmunized controls	Injected	70

Rainbow trout were injected intraperitoneally with 0.05 ml of vaccine made by adding β-propiolactone (1 : 6000) to a suspension containing $10^{7.3}$ $TCID_{50}$/ml IHNV. After 32 days, fish were challenged by subcutaneous injection of $10^{6.2}$ $TCID_{50}$ of wild type virus. The size of the fish at the time of immunization was not given.

A subunit vaccine derived from a cloned sequence of the glycoprotein gene[29] has been tested in the laboratory.[30] In trials using injected and water-borne delivery, protection against a water-borne challenge was obtained when vaccinates were compared with controls (Tables 15.4 and 15.5). This preparation has been shown to be capable of stimulating immunity in at least two species of host fish (rainbow trout and chinook salmon) and is effective against heterologous isolates (Table 15.5).

Table 15.3 *Immunization of rainbow trout with a formalin-killed vaccine against IHNV*[28]

Treatment	Challenge	Per cent mortality
Inoculated	Injected	5
Noninoculated controls	Injected	75
Immersed	Water-borne	50
Nonimmersed controls	Water-borne	80

Rainbow trout (2.5 g) were injected intraperitoneally with 0.05 ml of vaccine made from viral suspension containing 10×10^{10} $TCID_{50}$/ml inactivated with formalin (0.1 to 0.8 per cent) and challenged after 28 days by injection of $10 \times 10^{5.1}$ $TCID_{50}$/ml. Immersion vaccination of rainbow trout fry (0.9 g) was in a hyperosmotic solution containing 5 per cent inactivated vaccine in 5 per cent NaCl for 2 min. These fry were given a second immunization by immersion after 14 days. Two weeks after the booster immunization, these fish were challenged by immersion in $10 \times 10^{4.1}$ $TCID_{50}$/ml of wild type virus.

Table 15.4 *Immunization of rainbow trout with a subunit vaccine against IHNV*[30]

Treatment	Challenge	Per cent mortality
Inoculated[a]	Water-borne	9
Immersed[b]	Water-borne	36
Nonimmunized controls	Water-borne	60

[a]Rainbow trout (0.4 g) were injected with 0.01 ml of a bacterial lysate (3 mg/ml) containing 8 per cent expressed G protein.
[b]Rainbow trout (0.4 g) were immersed in the same bacterial lysate (50 fish per 10 ml of solution) for 3 min. After 1 month, fish were challenged by water-borne exposure to various concentrations of virus. Data shown are for a challenge with 3.2×10^3 $TCID_{50}$/ml.

Table 15.5 *Immunization of rainbow trout and chinook salmon with subunit vaccine against IHNV. Homologous* vs *heterologous challenge*[30]

Treatment	Challenge	Per cent mortality
Immersed	Round Butte IHNV[a]	19
Control	Round Butte IHNV[a]	92
Immersed	Elk River IHNV[b]	0
Control	Elk River IHNV[b]	64
Immersed	Dworshak IHNV[c]	11
Control	Dworshak IHNV[c]	85

[a]Rainbow trout (0.4 g) were immersed in a bacterial lysate (3 mg/ml) containing 8 per cent expressed G protein for 3 min. After 1 month, the fish were challenged by water-borne exposure to various concentrations of virus. Data shown are for a challenge of 7.2×10^5 $TCID_{50}$/ml.
[b]Chinook salmon (0.4 g) were immunized by immersion as above. The data shown here are for a challenge of 1.93×10^6 $TCID_{50}$/ml.
[c]Rainbow trout (0.4 g) were immunized by immersion as above. The data shown here are for a challenge of 2.85×10^3 $TCID_{50}$/ml.

No field trials with IHNV vaccines have been reported in the literature, but a test was conducted in California, USA using an attenuated strain of IHNV. The vaccine in this trial did not give adequate protection. Attenuated vaccines have also been tested in Idaho, USA with varying success.

Effectiveness

As side-by-side trials have not been reported, there is insufficient information to determine the comparative efficacy of the various types of IHN vaccines under development. However, differences in the effectiveness of each vaccine have been found to be dependent upon the method of delivery. When administered by immersion, modified live IHN vaccines have been effective and generally provide better immunity than an inactivated vaccine. This result may be expected because the replication of the attenuated virus stimulates additional forms of interference and immunity. This modified live vaccine has provided protection against both injected and water-borne challenge with wild type virus.[24]

When delivered by injection, killed vaccines have shown protection of fish against both types of challenge.[28] The subunit vaccine delivered by injection or immersion confers protection against a water-borne challenge.[30] The duration of the immunity and the effect of a booster dose on immunity has not been well established for any vaccine.

Limitations

The principal limitations for IHN vaccines involve safety, efficacy and cost. Attenuated vaccines must be avirulent and free of any potential for reversion via back mutation or recombination to a more virulent form. Reversion has been a concern for other live modified vaccines against rhabdoviruses (e.g. rabies). Both killed and subunit vaccines against IHNV administered by injection have demonstrated efficacy. However, this delivery method may prove too expensive for the vaccination of millions of small fish. Efficacy has also been demonstrated for the subunit vaccine when administered by immersion. The subunit vaccine will be less expensive to produce than a killed whole virus vaccine.

One potential limitation of all IHN vaccines may be the degree of cross-protection afforded fish against the biochemically and serologically disparate strains of IHNV that occur in different species of salmonids or in various geographic areas. If a cross-protection limitation is shown to be significant, the development of several IHN vaccines for use in each application (polyvalent preparations) will be required. Experimental studies, however, indicate that the recombinant subunit vaccine will confer protection against

challenge by at least two different isolates of IHNV (Table 15.5). More recently, we have shown that purified IHNV (Round Butte strain) glycoprotein, prepared from whole virus, will confer immunity to the five biochemically characterized IHNV strains (Engelking and Leong, unpublished results).

A final limitation may be the size of the commercial market. In order to defray the research and licensing costs necessary for the production of IHN vaccines, a sufficient market must be developed. The market will be determined by the efficacy and cost of the vaccine. Since in most applications millions of small and relatively inexpensive fish need protection, the cost per dose must be low. Also, the market will be restricted to portions of North America and Japan where IHNV is found.

Overcoming limitations

The limitations to the development of a safe, effective and inexpensive vaccine to protect salmonid fish against IHNV will be difficult to overcome. Important areas for research are the types of immunity stimulated by different viral vaccines, the use of adjuvants in enhancing immunity, and the role of booster immunizations. Attenuated vaccines will have to be proven stable and of low virulence, especially for the relatively small fish that must be protected against disease. The number of serotypes of IHNV must be determined. For inactivated and subunit vaccines, effective delivery systems to replace time-consuming injections should be developed.

For any of the IHNV vaccines under development or produced in the future, adequate markets must be assured to provide incentive for research and required licensing. Vaccines must have a low dosage cost and be easy to administer. Meeting these needs will require research and a better understanding of fish immunology.

Commercial prospects

In spite of the limitations of the vaccines currently being tested, the commercial development of an effective IHN vaccine appears promising. The regulatory process for the licensing of fish vaccines is improving, and salmonid aquaculture within the enzootic area is increasing. The spread of IHNV and its increased virulence for larger, more valuable trout will enlarge the market for any vaccine available.

The easiest vaccine to develop, test, and license may be a killed whole virus preparation, but the cost per dose would certainly be high. A subunit vaccine would be less expensive, and if efficiently delivered, relatively easy to license. The vaccine industry has expressed concern about licensing require-

ments for attenuated vaccines and their acceptance by fish pathologists, who rely on viral detection methods for certification of fish stocks, remains uncertain. Attenuated preparations also pose a problem of maintaining control for vaccine producers. Without a specific viral market and proper patent protection, it would be easy for anyone to purchase and produce the vaccine strain.

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MANUSCRIPT # 6

Expression in *Escherichia coli* of an Epitope of the Glycoprotein of Infectious Hematopoietic Necrosis Virus Protects against Viral Challenge

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EXPRESSION IN *ESCHERICHIA COLI* OF AN EPITOPE OF THE GLYCOPROTEIN OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS PROTECTS AGAINST VIRAL CHALLENGE

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Plasmid vectors were constructed that expressed an antigenic determinant of the glycoprotein gene of infectious hematopoietic necrosis virus (IHNV) as a fusion protein with the *trp*E protein of *Escherichia coli*. Insertion of Sau3AI fragments from the IHNV glycoprotein gene into *trp*E expression plasmids led to a fusion protein containing a hydrophilic segment of 104 amino acids from the middle portion of the viral glycoprotein. Although this region of the glycoprotein contains three N-linked glycosylation sites and is probably glycosylated in the virus, it is not glycosylated in bacteria. Nonetheless, immunization trials in fish with the crude bacterial lysate containing the fusion protein showed that the *trp*E-glycoprotein fusion protein produced in bacteria induced protective immunity.

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus endemic to the North American northwest and causes a fatal disease in salmon and trout[1]. The virus is transmitted horizontally through the water from infected fish[18] and vertically by virus contamination of the surface of infected eggs[19]. IHNV is characterized by its bullet-shaped morphology, enveloped nucleocapsid and glycoprotein projections[2]; its RNA genome is of negative polarity and is approximately 11,000 bases encoding the five virion proteins, N (nucleocapsid), M1 and M2 (matrix proteins), G (glycoprotein), and L (polymerase)[7,15]. In addition, IHNV transcribes a sixth mRNA for a nonvirion associated protein, NV, of unknown function; a feature not described for other rhabdoviruses[12,13].

Recent studies in our laboratory have shown that the glycoprotein is the only antigen capable of eliciting a neutralizing antibody response against the virus *in vitro* (Engelking and Leong, manuscript submitted). Further experiments have shown that purified virion glycoprotein is capable of producing a protective response in salmonid fishes to a lethal virus challenge (Engelking and Leong, manuscript submitted). Currently, there is no vaccine to control outbreaks of IHNV, which results in severe economic damage to the aquaculture industry. Development of a subunit vaccine produced by the expression in *E. coli* of a viral epitope immunoreactive with IHNV neutralizing antisera was undertaken and the results are described here.

A viral epitope(s) of the IHNV glycoprotein was expressed as a fusion protein with the *trp*E protein of *E. coli*. This nonglycosylated fusion protein contained a hydrophilic segment of 104 amino acids from a region of the glycoprotein, which is potentially glycosylated in the virus. Despite the lack of carbohydrate side chains, the fusion protein was effective in inducing protective immunity in fish.

RESULTS

Construction of *trp*E-G expression plasmid. We have shown that purified IHNV-G protein will elicit a protective immune response in salmonid fry (Engelking and Leong, manuscript submitted). The objective of this study was the insertion of the IHNV glycoprotein gene into a suitable *E. coli* expression vector and the subsequent use of the expressed protein as an antiviral vaccine. Previous work with the rabies virus glycoprotein gene expressed in bacteria[17,24] indicated that the expressed glycoprotein was denatured in bacteria and did not produce a protective immune response. A cloning strategy was therefore employed that might circumvent this problem for the IHNV-G protein. Recombinant plasmids containing fragments of the G gene were generated rather than plasmids containing the intact G gene. Only those recombinant plasmids expressing an epitope of the G protein would be detected upon screening with anti-G protein specific serum.

Examination of the nucleotide sequence of a cDNA clone of the IHNV-G mRNA indicated that Sau3AI digestion would result in nine fragments ranging in size from 40 to 329 bp. The isolated G cDNA insert from pG8 was digested with Sau3AI, and a mixture of these fragments was randomly ligated to the BamHI site of the pATH3 expression vector (Fig. 1).

Transformant colonies were screened for IHNV-G epitope expression with anti-IHNV serum. Positive colonies were detected with pATH3 as the expression vector. For subsequent analysis, twelve positively staining colonies were selected. The presence of viral G gene information in the recombinant plasmids carried by these colonies was confirmed by colony blot DNA hybridization (data not shown).

Plasmids were isolated from individual immunopositive colonies and digested with EcoRI and HindIII. An estimate of each insert's size was determined by agarose gel electrophoresis of the digested plasmid. The results indicated that there were only two different insert sizes, approximately 600 and 1800 bp, among the twelve selected colonies. The large size of these inserts indicated that two or more Sau3AI fragments were ligated together during the cloning procedure, a result confirmed by DNA sequence analysis of the inserts. Representatives of the two groups, p52G for the smaller insert and p618G for the larger insert, were used in the studies described below.

Expression of *trp*E-G fusion protein. Individual immunopositive colonies were grown in broth culture and

induced with indoleacrylic acid. Bacterial lysates were prepared and the proteins were analyzed by SDS-PAGE. Coomassie Blue staining of these gels indicated that there were two groups of immunopositive colonies. One group, represented by p618G, produced a *trp*E-fusion protein with an apparent molecular weight of 48,000 Daltons that was easily detected in the gel (Fig. 2A). The second group, represented by p52G, produced a fusion protein that was only detected with Western blotting (Fig. 2B). The fusion protein expressed in this group had an apparent molecular weight of 49,000 Daltons and was masked on Coomassie Blue stained gels by other bacterial proteins. Some lower molecular weight minor bands were also detected in lanes f and j by Western blotting (Fig. 2B). One explanation for the presence of these bands is proteolytic degradation of the cloned product; rapid degradation of exogenous proteins has been reported for *E. coli*[16]. These bands were absent from control lanes g and i (Fig. 2B). Extended incubation of the cells or storage of lysates at either 4° or −20°C did not change the pattern or formation of the smaller bands. Laser densitometric scanning of the Coomassie Blue stained gel of the bacterial lysate of p618G showed that the fusion product made up approximately 10% of the total cell protein.

The *trp*E-glycoprotein fusion was purified from a preparative SDS-polyacrylamide gel and used to immunize rabbits. After adsorption with control *E. coli* cells containing the pATH3 vector alone, the antiserum reacted with purified IHNV as shown by enzyme linked immunoassay and weakly neutralized IHNV in plaque assays. The antiserum also detected the IHNV glycoprotein in Western immunoblots of purified virus (data not shown).

Sequence determination of the viral insert. Examination of the IHNV glycoprotein nucleotide sequence[11] indicated that only one Sau3AI fragment could be inserted in the correct reading frame in pATH3. This was a 329 bp fragment from the approximate carboxyl third of the IHNV glycoprotein gene. DNA sequence analysis verified this by demonstrating that in both p52G and p618G, the Sau3AI-329 bp fragment was inserted in frame with the structural gene for *trp*E. The 329 bp fragment encoded a peptide of approximately 11,000 Daltons. Thus, the *trp*E-G fusion protein of 49,000 Daltons contained 37,000 Daltons of the *trp*E polypeptide and the additional 11,000 Dalton G protein fragment. Due to the "shotgun" approach of cloning the nine Sau3AI fragments randomly into each expression vector, there was the potential for the fragments to be ligated together as well as into the vector. DNA sequence examination of these positive expression plasmids showed that extraneous Sau3AI fragments did indeed ligate tandemly. However, as observed from the Western blot, even though these plasmids differed greatly in size, their respective fusion proteins were approximately equal, indicating that the extra fragments were not in frame and therefore not translated. A determination of the DNA sequence of p52G indicated that the 264 bp Sau3AI fragment (Fig. 1) was ligated adjacent to the 329 bp fragment and the termination codon TAG is found 84 bp from the end of the 329 bp fragment. The 593 bp fragment provided genetic information for a peptide of 49,000 Daltons (49kD=37kD [*trp*E] + 11kD [Sau3AI-329] + 1 kD [84 bp extra]). Determination of the DNA sequence of p618G indicates that the 100 bp Sau3AI fragment (Fig. 1) is ligated adjacent to the 329 bp fragment and the termination codon TGA is found 6 bp from the end of the 329 bp fragment, thus accounting for the approximate 1000 Dalton difference in fusion protein size between p52G and p618G. DNA sequencing past the Sau3AI-100bp fragment in p618G was not performed.

The Sau3AI-329bp fragment was located in the carbox-

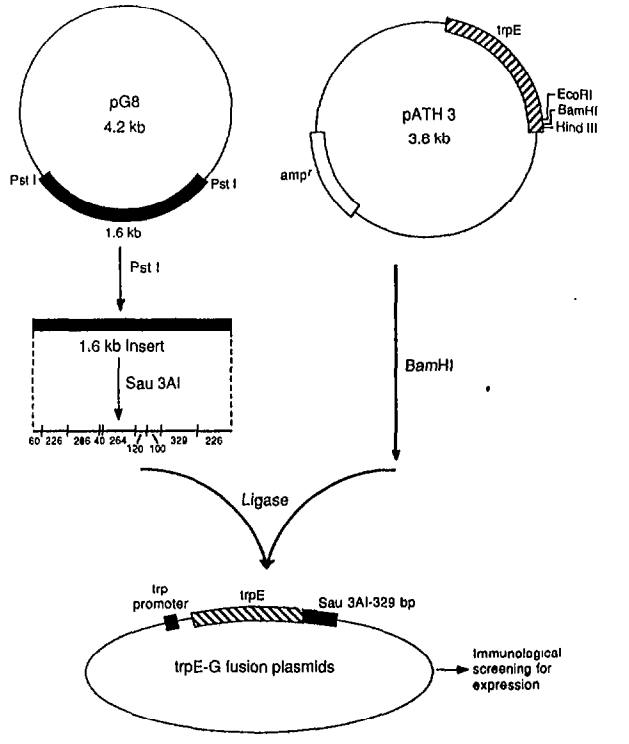

FIGURE 1 Construction of *trp*E-G gene fusions. The 1.6 kb cDNA cloned insert of the IHNV glycoprotein gene was restricted with Sau3AI and subcloned as described in Experimental Protocol. The resulting plasmids used for expression analyses, p52G and p618G, were generated.

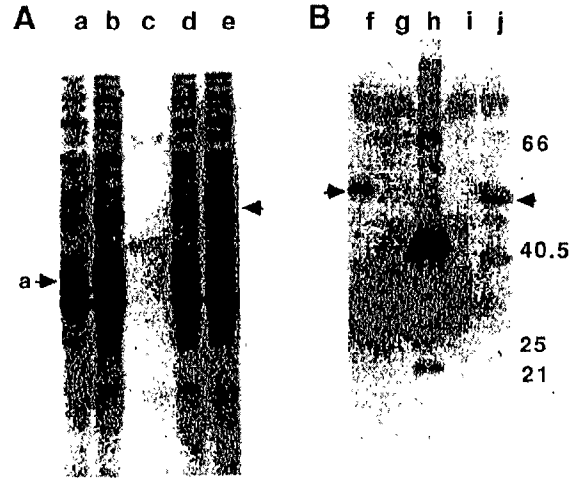

FIGURE 2 Analysis of bacterial production of *trp*E-G fusion protein on 10% SDS-polyacrylamide gel and antibody reactivity on electrophoretic transfer blot. (A) Coomassie Blue stained gel of proteins prepared from bacteria with various plasmids. Lanes: a, proteins of cells containing plasmid 52G; e, proteins of cells containing plasmid 618G; b and d, proteins of cells containing pATH3; c, IHNV structural proteins showing G at 66kd and N at 40.5kd. Arrow *a* indicates the position of *trp*E protein in cells containing the pATH3 vector alone. The solid arrow next to lane e indicates the *trp*E-G fusion protein in that lane. (B) Electrophoretic blot of protein fractions in (A). Lanes a through e were analyzed with rabbit antisera produced against IHNV and are shown as lanes f through j. Lanes f and j show the *trp*E-G fusion protein (arrows) of plasmids 52G and 618G respectively. The major band for p52G migrated at a position of 49,000 and for p618G at 48,000 Daltons. Lanes g and i contain the protein samples from cells containing the pATH3 vector alone. Lane h contains the IHNV structural proteins with G at 66 kD, N at 40.5 kD, m1 at 25 kD, and M2 at 21 kD.

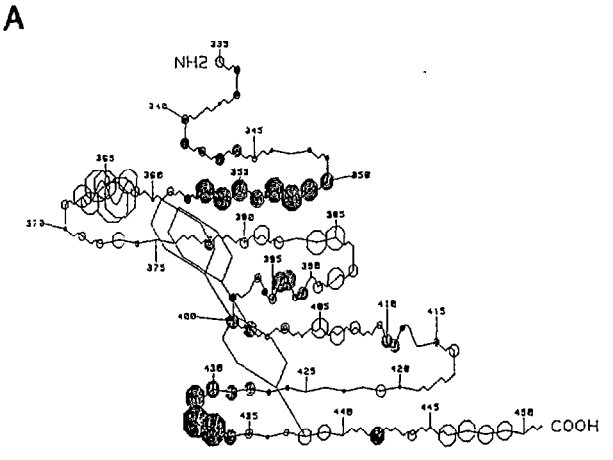

FIGURE 3 Predicted secondary structure and hydropathy of the IHNV glycoprotein gene region cloned in pATH3. (A) Open circles indicate hydrophilic regions and shaded circles indicate hydrophobic areas. The radius of a circle over a residue position is proportional to the average hydrophilicity or hydrophobicity as calculated for that residue and the next five residues. Numbers correspond to amino acid residues 335 to 450. The Sau3AI-329 bp fragment present in p52G and p618G encodes amino acid residues 336 to 444. The large open hexagonal structures point to predicted N-glycosylation sites at asparagine residues 400, 401, and 438. Rapid zig-zag lines indicate β sheets with α-carbons alternating above and below the chain. Random coils are represented as gently undulating straight lines and α-helices are shown as sine waves. This model of the antigenic region was generated by E. Golub, University of Pennsylvania[23]. (B) Amino acid sequence of Sau3AI-329 bp fragment.

yl terminal third of the coding region of the IHNV glycoprotein gene (Fig. 1) just before the transmembrane domain. An examination of a hydropathy plot of the deduced amino acid sequence of this region indicated that the glycoprotein region expressed in the fusion protein was very hydrophilic[11] (Fig. 3).

Immunization of fish with crude fusion protein. Immunization of rainbow trout and chinook salmon fry with a lysate prepared from bacteria containing p52G and/or p618G was performed as described, and subsequent viral challenges provided data on the efficacy of the fusion protein as a vaccine (Tables 1 and 2).

Table 1 shows the significant protection conferred on immunized fish versus unimmunized fish when challenged with the Round Butte isolate of IHNV. The glycoprotein gene used in constructing the fusion protein was derived from this strain. Cross protection against a more virulent isolate of IHNV, Dworshak, was also shown. A significant decrease in mortalities from 50 to 70 per cent occurred in the immunized fish at the higher virus dilutions in this challenge experiment. The statistical significance of the difference in mortalities at each virus dilution was confirmed by logistic regression analysis (analyses not shown).

A group of fish immunized with bacterial lysate derived from *E. coli* that contained the pATH3 vector with no insert was used in a challenge experiment to determine if nonspecific immunity was induced. Table 2 shows that in the mock immunized group, the mortality rate was essentially the same as in the non-immunized control group. The similarity of response between mock, immunized and control untreated fish was confirmed by estimation of logistic regression models. In addition, the immunized groups show cross protection when challenged with another IHNV isolate, Elk River, and in a different salmonid species, the chinook salmon.

TABLE 1 Immunization trials using *E. coli* expressed fusion product. Comparison of immersed vaccinated rainbow trout fry to unvaccinated control fry against challenge with IHNV Round Butte and Dworshak isolates.

IHNV Round Butte strain vs. rainbow trout						
	Immunized p52G			Control		
Virus dilution*	No.	SL	%	No.	SL	%
−2	25	5	20	25	23	92
−3	26	4	15	25	18	72
−4	26	0	0	25	15	60
−5	26	0	0	25	4	16
IHNV Dworshak strain vs. rainbow trout						
	Immunized p52G			Control		
Virus dilution**	No.	SL	%	No.	SL	%
−2	28	26	93+	24	24	100
−3	26	20	77+	27	25	93
−4	27	14	52	26	26	100
−5	27	3	11	26	22	85

No. = Number of fish in group.
SL = Specific loss, i.e., number of fish dying from IHNV infection.
% = Percent mortality.
* = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 7.2×10^7 $TCID_{50}$/ml.
** = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 2.8×10^8 $TCID_{50}$/ml.
\+ = Not statistically different from control.

DISCUSSION

Infectious hematopoietic necrosis virus is very similar to rabies virus in its virion protein pattern[15] and rabies virus has often been viewed as prototypic for IHNV studies. Work on the rabies glycoprotein demonstrated that the purified glycoprotein was highly immunoprotective and similarly, we have shown that purified IHNV glycoprotein will also induce a protective immune response in fish (Engelking and Leong, manuscript submitted). These initial findings indicated that both viruses would be ideal for the application of recombinant DNA techniques to develop a subunit vaccine. In fact, an inexpensive IHNV subunit vaccine is the only economically feasible prophylactic for the aquaculture industry. When a full length cDNA clone of the mature rabies virus glycoprotein gene was expressed in bacteria[17,24], the product did not elicit a

protective immune response in mice[24]. This lack of immunocompetency was thought to result from the fact that cytoplasmic proteins in *E. coli* are made in a reduced state and stable disulfide bonds are not formed[16].

In order to avoid the problems encountered with the rabies glycoprotein expression in *E. coli*, only a small region of the IHNV glycoprotein gene was expressed as part of a *trp*E fusion protein. The simultaneous ligation of all Sau3AI fragments into the expression vector enabled us to quickly and efficiently express in bacteria a region(s) of the gene that encoded an immunogenic determinant. Portions of the IHNV glycoprotein gene were attached to the *trp*E operon promoter and a portion of the *trp*E gene of *E. coli* to generate fusion proteins. The transformant colonies were screened for antigen production by immunologically assayed colony blots and thus, only those fusion constructions that resulted in the expression of an antigenic domain were selected. These procedures resulted in the identification of a Sau3AI fragment of 329 bp, which encoded a protein domain recognized by the polyclonal rabbit anti-IHNV glycoprotein serum.

An examination of the deduced amino acid sequence and the hydropathy of the Sau3AI-329 bp fragment indicated that this region of the glycoprotein gene contained a sequence of very hydrophilic amino acids from residue 361 to 369. This hydrophilic domain protrudes at a predicted beta turn in the glycoprotein chain[11] and may appear as an epitope in the native molecule (Fig. 3). There in only one cysteine residue at position 357 among the 104 amino acids encoded by the Sau3AI-329 bp fragment and yet this region is still immunocompetent in fish (Fig. 3B). These results indicate that intramolecular disulfide bonds are not required to maintain the immunogenic structure of the protein in this region of the molecule.

The cloned region also contains three predicted N-linked glycosylation sites at positions 400, 401, and 438[11]. Since eukaryotic proteins are not glycosylated in *Escherichia coli*, it is clear that the absence of glycosylation did not affect the recognition by antisera of this region of the G protein. Altered glycosylation sites have been reported in rabies variants selected by resistance to neutralizing monoclonal antibodies[23]. This work suggested that glycosylation was important in altering epitope recognition for virus neutralization. However, the results reported here show specifically that a small region of approximately 100 amino acids remains antigenically recognizable without glycosylation and antibody produced in rabbits to the fusion protein is neutralizing (data not shown).

There are two other *trp*E fusion protein expression vectors in the pATH series, pATH1 and pATH2 (Koerner and Tzagaloff, personal communication). All three vectors together provide restriction sites that permit the insertion of a DNA sequence in the three available reading frames. An analysis of other possible antigenic determinants of the IHNV glycoprotein is being made by using pATH1 and pATH2 as vectors for the expression of different regions of the glycoprotein.

The *trp*E glycoprotein fusion protein was tested in immunization trials in fish. The crude protein lysate from a 1 liter culture was used to immunize 100 fish which were approximately 2 months old and less than 0.5 g in size. A protective immune response was induced in fish to three different isolates of IHNV and indicated that the peptide encoded by the Sau3AI-329 bp fragment of the G gene contained an epitope(s) common to all three virus isolates. An estimate of the LD_{50} (*L*ethal *D*ose for 50% of the animals) indicated that approximately 1000 times more virus was required to kill the vaccinated fish as compared to control fish. The fish were challenged with virus levels ranging from thousands to millions of tissue culture infectious virus units/ml water. In the hatchery environment, the fish can be exposed to virus levels that range from 1 to 0.1 infectious units/5 ml of water[22]. Virus levels as high as 32.5 to 1600 units/ml of water have measured in water where spawning fish are held at high densities[18]. The vaccine did protect fish at virus concentrations in excess of these reported levels.

A comparison of the immunogenicity of the recombinant vaccine and purified IHNV glycoprotein suggested that recombinant vaccine was much more effective in inducing protective immunity. The average amount of virus required to achieve an LD_{50} in fish immunized with the purified glycoprotein was 54–166 times that required for unimmunized controls (Engelking and Leong, unpublished data). This is in marked contrast to the recombinant vaccine where 600–1000 times more virus was required to achieve an LD_{50} as compared to unimmunized control fish. The apparent enhanced immunogenicity of the recombinant vaccine may result from adjuvant active cell wall components that remain in the vaccine preparation.

Previous studies of immunization in fish have indicated that the minimum size for successful immunization by immersion was 0.8 g for chinook salmon[5] and 1–2.5 g for rainbow trout[9]. In fact, these investigations have shown that size and not age of the fish was important in the development of an immune response upon vaccination[9]. The immunization trials reported here were carried out on chinook salmon and rainbow trout fry that were 0.5 g in size. These fish were able to respond effectively to the viral vaccine. Since these fish were so small, it was not possible to develop an accurate and reliable assay for the anti-IHNV antibody response in the vaccinated animals. Under optimal conditions only 5–10 µl of blood were obtained from fish of this size. The duration of this

TABLE 2 Immunization trials using *E. coli* expressed fusion product. Comparison of immersed vaccinated chinook salmon fry to unvaccinated control fry against challenge with IHNV Elk River isolate.

IHNV Elk River strain vs. chinook salmon												
Virus dilution*	Immunized p52G			Immunized p618G			Mock vaccinated control			Unvaccinated control		
	No.	SL	%	No.	SL	%	No.	SL	%	No.	SL	%
−2	28	2	7	29	0	0	19	10	53	25	16	64
−3	28	2	7	29	0	0	19	4	21	25	6	24
−4	28	0	0	29	3	10	18	3	17	25	3	12
−5	29	0	0	30	2	7	17	1	6	25	5	20

No. = Number of fish in group.
SL = Specific loss, i.e., number of fish dying from IHNV infection.
% = Percent mortality.
* = Virus dilutions are shown as ten-fold dilutions of a stock virus with a titer of 1.9×10^8 $TCID_{50}$/ml.

immunity was not determined.

The development of any vaccine must have safety as well as efficacy as one of its primary considerations. The safety of live attenuated vaccines has been questioned for the aquaculture industry because of the very nature of the environment where the vaccine would be applied. The vaccine has to be completely safe for cultured and wild salmonid fish in the watershed. Moreover, the vaccine has to be economical and a subunit vaccine produced in bacteria seems to be a viable alternative. The initial trials of the subunit vaccine reported here suggests that the *trp*E fusion protein, even in a crude lysate, can be utilized as an effective and economical vaccine against IHNV.

EXPERIMENTAL PROTOCOL

Cells and virus. The IHNV isolates Elk River, Dworshak and Round Butte used in these studies were obtained from W. Groberg (Oregon Department of Fisheries and Wildlife) and have been described[8]. The virus used for challenge studies was prepared by infecting rainbow trout (*Salmo gairdneri*) fry and reisolating the virus from fish dying of IHN disease. Subsequently, the virus was grown for two passages in chinook salmon embryo cells, CHSE-214[4].

Construction of recombinant plasmids. The construction of a recombinant plasmid containing the *trp*E promoter and gene fused to a fragment of the IHNV glycoprotein gene is shown in Figure 1. Plasmid pG8 contains the entire coding sequences for the glycoprotein from IHNV isolated from Round Butte Hatchery, Oregon[11]. The 1.6 kb glycoprotein gene insert of pG8 was excised by cleavage with PstI and purified by gel electrophoresis in low melting temperature agarose (SeaKem, FMC Corporation Marine Colloids Div.). The purified fragment, digested with restriction endonuclease Sau3AI, yielded 9 fragments ranging in size from 40 to 329 basepairs (bp). These fragments were ligated to the pATH3 *trp*E fusion protein expression vector previously cleaved with BamHI in a T4 DNA ligase reaction[18]. The entire mixture was used to transform *E. coli* strain MC1061[5] and transformants were identified by colony blot hybridization. Transformants were grown on Luria-Bertani (LB) agar plates containing 120 μg/ml ampicillin. The pATH3 vector was the generous gift of T. J. Koerner and A. Tzagaloff (Columbia University). The constructions were verified by DNA sequence analysis by the dideoxy method[20].

Immunologic detection of viral peptide protein expression. Transformants were analyzed for viral peptide production by immunologic detection. The transformant colonies were transferred to nitrocellulose by replica plating[6] and the nitrocellulose was overlayed on minimal media M9 containing 1% casamino acids, 10 μg/ml indoleacrylic acid, and 120 μg/ml ampicillin[18]. The cells were allowed to grow overnight at 37°C; then lysed by exposure to chloroform vapor for 2 h and subsequently, the filters were analyzed for viral antigen with rabbit anti-IHNV and horseradish peroxidase conjugated goat anti-rabbit immunoglobulin serum as previously described[8].

Western immunoblotting. The insoluble *E. coli* lysates from positive cultures were prepared as described[10]. Proteins were analyzed by sodium dodecyl sulfate gel electrophoresis (SDS PAGE) on a 10% polyacrylamide gel by the discontinuous gel method of Laemmli[14]. The proteins distributed on the gel were electrophoretically transferred to 0.2 μm nitrocellulose[21]. The Western blots were developed with rabbit anti-IHNV serum and rabbit antiserum prepared against purified IHNV glycoprotein (Engelking and Leong, manuscript submitted).

Immunization trials in fish. Bacterial crude lysates were prepared as described[10] and used to immunize fish by immersion. A set of approximately 100 rainbow trout or chinook salmon (*Oncorhynchus tshawytscha*) fry were used for each control and immunized experimental group. The average weight of each fry at the time of immunization was 0.4 g. Immunization by immersion was accomplished by bathing groups of 100 fry in 25 ml of the preparation (ca. 3 mg/ml total protein concentration) for 1 min. At that time the immersion solution volume was increased to 250 ml with water and fish were incubated in this diluted solution for an additional 2 min. These fish were then placed in aquaria of 5 gallons with a water flow rate of 0.25 gal/min in a constant water temperature of 10°C. Approximately one month after immunization, each fish group was subdivided and challenged with varying doses of live, virulent IHNV. All dead fish were assayed for the presence of infectious IHNV in chinook salmon cells (CHSE-214) as described by Engelking and

Leong[4]. The data was analyzed statistically by estimation of logistic regression models.

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MANUSCRIPT # 7

The Nucleocapsid Gene of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus

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The Nucleocapsid Gene of Infectious Hematopoietic Necrosis Virus, a Fish Rhabdovirus[1]

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The complete nucleotide sequence of the infectious hematopoietic necrosis virus (IHNV) nucleocapsid gene has been determined using cDNA clones of genomic and messenger RNAs. Genomic clones were generated by using random DNA oligomers to prime cDNA synthesis and were mapped to their respective locations on the genome by the use of cDNA probes derived from viral mRNAs. Interesting features of the IHNV nucleocapsid gene sequence elucidated by the sequencing of these clones include short homologies with N genes of other rhabdoviruses at the 5′ and 3′ nontranslated termini of the mRNA, as well as an exceptionally long 5′ noncoding region of the mRNA, suggesting a leader RNA may be coupled to the N mRNA. A comparison of the IHNV N protein coding sequence with other rhabdoviral N genes shows some homologies at the amino acid level which indicates the possible evolutionary relationship of these N proteins. The determination of the nucleotide sequences of IHNV genes and intergenic regions will be useful for studying the mechanisms of rhabdoviral transcription and replication.

Infectious hematopoietic necrosis virus (IHNV) belongs to the family Rhabdoviridae and infects salmonids, usually causing a fatal disease (*1*). IHNV is similar to other rhabdoviruses with its bullet-shaped structure and negative-sense, single-stranded RNA genome of approximately 11,000 bases (*2–5*). The virion consists of a polymerase (L), two matrix proteins (M1 and M2), a surface glycoprotein (G), and a nucleocapsid protein (N) (*6*). IHNV differs from the mammalian prototype rhabdoviruses, vesicular stomatitis virus (VSV), and rabies in having an optimal growth temperature of 12–15° in its piscine host and in having a slow growth rate which produces low yields of progeny virus (*7*). Another significant difference from other rhabdoviruses previously studied is that IHNV produces six monocistronic mRNAs rather than five during transcription (*5*). Five of these mRNAs encode the viral structural proteins and a sixth mRNA encodes a unique nonvirion protein, NV, whose function is not yet known (*5*).

The physical map of the virus genome has been determined (*8*), an *in vitro* transcription system for IHNV has been described (*9, 10*), and the sequence of the G gene mRNA has been reported (*11*). However, the molecular mechanisms in the transcription and replication of IHNV and the regulatory sequences governing these processes have yet to be determined. Therefore, to further these studies, we have undertaken the cloning of the IHNV genome by using random DNA oligomers to prime cDNA synthesis and generate clones that span the entirety of the genome. We report in this paper the complete nucleotide sequence of the N gene including the observation of a long (ca. 100 bases) noncoding region on the 5′ end. This "leader" region was present in both an mRNA and a genomic clone. We speculate that this region may be similar to a leader RNA found in other rhabdoviruses.

The cDNA clones of genomic fragments were generated using calf thymus primers and the plasmid vector, pUC8, as previously described (*5*). After an initial plasmid screening, large inserts were mapped to their corresponding genomic location by using the mRNA clones of known genomic position (*8*). Four recombinant clones that span the entire N gene were identified. These clones, p470, p522, p292, and p163, had insert sizes of 750, 615, 800, and 750 bp, respectively. The orientation of these cloned inserts on the viral genome were established by specific hybridization to pN410, a mRNA cDNA clone from the 5′ end of the gene, and pN144, a mRNA cDNA clone from the 3′ end of the gene (Fig. 1B) (*8*). The nucleotide sequence of the N gene was determined by using the mRNA clones already described and the genomic clones. A partial restriction map was constructed by digestion of the N gene cloned inserts with an array of restriction enzymes, and the sequencing strategy by the dideoxy chain termination method (*12*) in M13 (*13*) is shown in Fig. 1B.

The nucleotide sequence of the IHNV N gene in which nucleotides are numbered relative to an arbi-

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. J04321.

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1
AGT CAC GGA GAT AAC GAT CTT CAG ACA CTA TAA ACC GAG ACA GAA CAA GCA GAA CTA TTT TCA CTG AAA ACA ACA CCT GAG AGA CAG AAA CGG ATC A
                                  UU UGG
101
 CG AAC G ATG ACA AGC GCA CTC AGA GAG ACG TTC ACT GGA CTC AGA GAC ATC AAG GGG GGA GTC CTC GAG GAT CCA GAG ACG GAG TAT CGT CCC GGT ACG A
          MET Thr Ser Ala Leu Arg Glu Thr Phe Thr Gly Leu Arg Asp Ile Lys Gly Gly Val Leu Glu Asp Ala Glu Thr Glu Tyr Arg Pro Gly Thr      31
201
 TA ACC CTC CCT CTC TTT TTC TCC AAG GCA GAC TTT GAC CTA GAG ATG ATC AAG CGG GCG GTG AGT CAC GTC GGA GGA GAG GGA ACG AGA AGG GCA TTG GG
Ile Thr Leu Pro Leu Phe Phe Ser Lys Ala Asp Phe Asp Leu Glu MET Ile Lys Arg Ala Val Ser His Val Gly Gly Glu Gly Thr Arg Arg Ala Leu Gly  65
301
  C CTC CTG TGC GCG TTC GTC ATT GCA GAG ACG GTC CAT CGG GGA GAG GCA CGG TCG CCG AAC TTC TGG GCT TCT TGC TGG AGT CTT TGG AGT CTT TGG AGA
    Leu Leu Cys Ala Phe Val Ile Ala Glu Thr Val His Arg Gly Glu Ala Arg Ser Pro Asn Phe Trp Lys Pro Trp Ala Ser Cys Trp Ser Leu Trp Thr  98
401
CTG GGG CAC CAC TGG AAC GTT ACC TTC GCA GAT CCC AAC AAC AAG CTT GCA GAA ACG ATC GTA AAG GAA AAT GTC CTT GAG GTT GTG ACC GGC CTC CTC T
Leu Gly His His Trp Asn Val Thr Phe Ala Asp Pro Asn Asn Lys Leu Ala Glu Thr Ile Val Lys Glu Asn Val Leu Glu Val Val Thr Gly Leu Leu  131
501
 TC ACC TGC GCC CTA CTG ACA AAG TAT GAT GTG GAC AAG ATG GCC ACA TAC TGC CAA AAC AAG CTC GAG CGT CTT GCA ACC AGC CAA GGG ATT GGC GAG TT
  Phe Thr Cys Ala Leu Leu Thr Lys Tyr Asp Val Asp Lys MET Ala Thr Tyr Cys Gln Asn Lys Leu Glu Arg Leu Ala Thr Ser Gln Gly Ile Gly Glu Leu  165
601
  G GTC AAC TTC AAC GCC AAC AGG GGA GTG CTG GCC AGG ATC GGG GCG GTA CTT AGA CCT GGA CAG AAG CTC ACC AAG GCT ATC TAT GGG ATC ATT CTC ATC
    Val Asn Phe Asn Ala Asn Arg Gly Val Leu Ala Arg Ile Gly Ala Val Leu Arg Pro Gly Gln Lys Leu Thr Lys Ala Ile Tyr Gly Ile Ile Leu Ile  198
701
AAC CTG TCC GAC CCA GCC ACC GCC GCT AGA GCC AAG GCA CTG TGC GCC ATG AGA CTG AGC GGG ACA GGA ATG ACA ATG GTG GGA CTG TTC AAC CAA GCC G
Asn Leu Ser Asp Pro Ala Thr Ala Ala Arg Ala Lys Ala Leu Cys Ala MET Arg Leu Ser Gly Thr Gly MET Thr MET Val Gly Leu Phe Asn Gln Ala  231
801
 CA AAG AAC CTG GGC GCC CTT CCA GCC GAC CTT CTA GAA GAT CTG TGC ATG AAG TCA GTG GTG GAG TCC GCC AGA CGC ATT GTC AGA CTG ATG AGG ATC GT
  Ala Lys Asn Leu Gly Ala Leu Pro Ala Asp Leu Leu Glu Asp Leu Cys MET Lys Ser Val Val Glu Ser Ala Arg Arg Ile Val Arg Leu MET Arg Ile Val  265
901
  A GCA GAG GCC CCA GGG GTA GCA GCA AAG TAC GGT GTC ATG ATG AGC AGG ATG CTC GGG GTG GGG TAC TTC AAG GCC TAC GGG ATC AAC GAG AAC GCC AGG
    Ala Glu Ala Pro Gly Val Ala Ala Lys Tyr Gly Val MET MET Ser Arg MET Leu Gly Val Gly Tyr Phe Lys Ala Tyr Gly Ile Asn Glu Asn Ala Arg  298
1001
ATC ACC TGC ATT CTC ATG AAC ATC AAC GAT AGG TAT GAC GAT GGG ACC TCG GGA GGA CTG ACA GGG TTG AAG GTA TCC GAC CCT TTC AGG AAG CTG GCG A
Ile Thr Cys Ile Leu MET Asn Ile Asn Asp Arg Tyr Asp Asp Gly Thr Ser Gly Gly Leu Thr Gly Val Lys Val Ser Asp Pro Phe Arg Lys Leu Ala  331
1101
 GA GAG ATC GCT CGT CTC CTT GTC CTC AAG TAC GAC GGC GAT GGC TCA ACC GGA GAG GGG GCG TCC GAC CTG ATC CGC CGG GCG AGA TGG CAT CCC GGG GA
  Arg Glu Ile Ala Arg Leu Leu Val Leu Lys Tyr Asp Gly Asp Gly Ser Thr Gly Glu Gly Ala Ser Glu Leu Ile Arg Arg Ala Arg Trp His Pro Gly Asp  365
1201
  C CAG ACA TGG GTG AGG AGG AGG AGG AGG ACG AGG AGG ACG ACG ACT CCA GTG AGC CAG GAG ACT CCG ATT CAT TCC TCT GAA CAC CAA CAA CCC CCT CTT
    Gln Thr Trp Val Arg Arg Arg Arg Arg Thr Arg Arg Thr Thr Thr Pro Val Ser Gln Glu Thr Pro Ile His Ser Ser Glu His Gln Gln Pro Pro Leu 398
1301
CTC CCG CCC CTC GAC CCA TCC AGA CGT TTA CTC CAC CCT GAC TCC TAG AT AGA AAA AAA
Leu Pro Pro Leu Asp Pro Ser Arg Arg Leu Leu His Pro Asp Ser TER                  413
```

B.

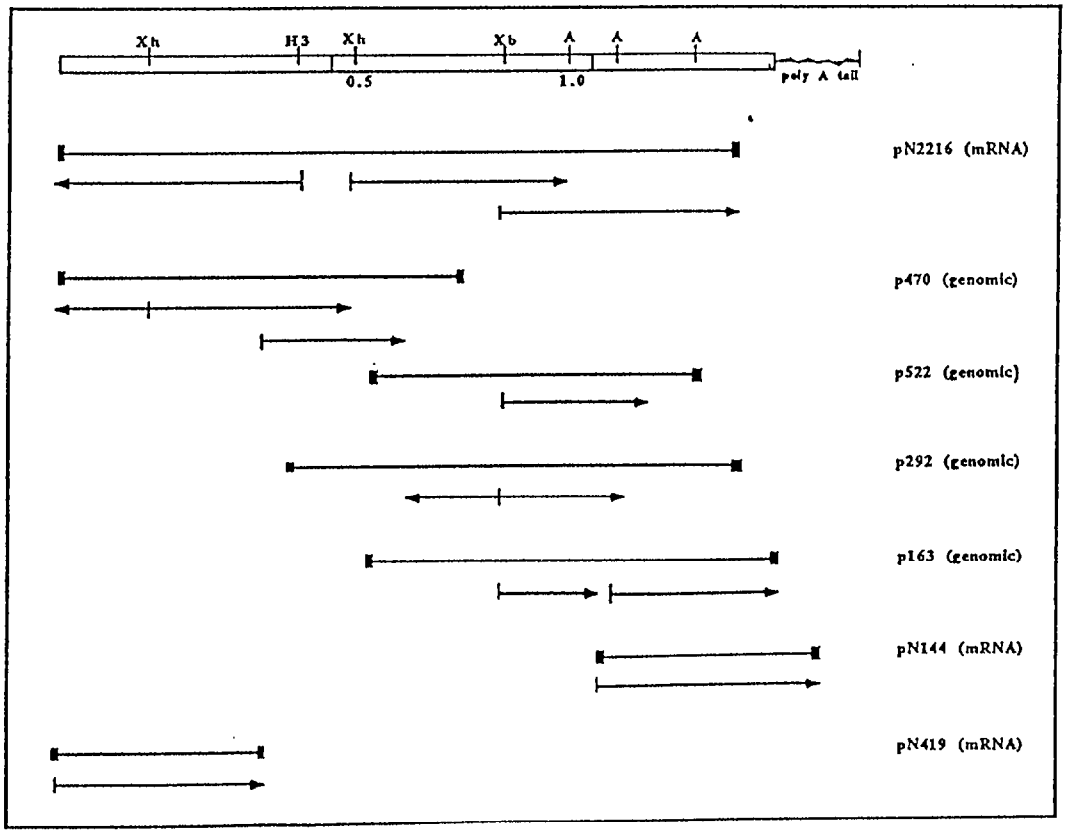
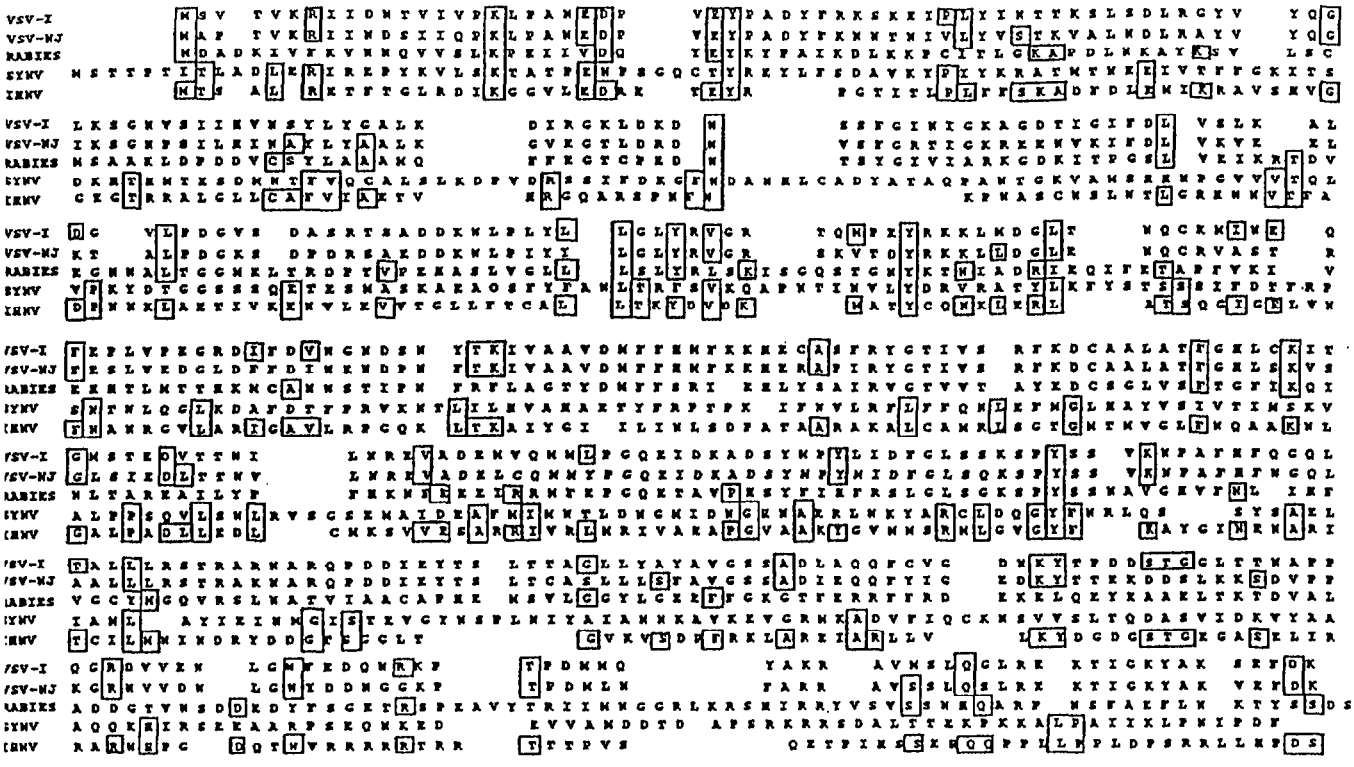


FIG. 1. Nucleotide sequence and sequencing strategy for the IHNV N gene. (A) The nucleotide sequence of the IHNV N gene is shown in the mRNA (+) sense along with the deduced amino acid sequence of the continuous open reading frame beginning with the Met codon at position 107 and terminating with TAA at position 1346. The dots denote the nucleotide sequence 5′-AAACC-3′ in the plus strand or 3′-UUUGG-5′ in the viral genome, which is commonly found in rhabdovirus leader RNAs. (B) The physical map and sequencing strategy for the IHNV N protein gene is depicted with double lines representing a partial restriction map of the N gene, thin lines bounded by arrows indicating the extent and direction of the nucleotide sequencing, and thin lines bounded by boxes representing the genomic or mRNA cDNA clones used in the analysis.

A.



B.

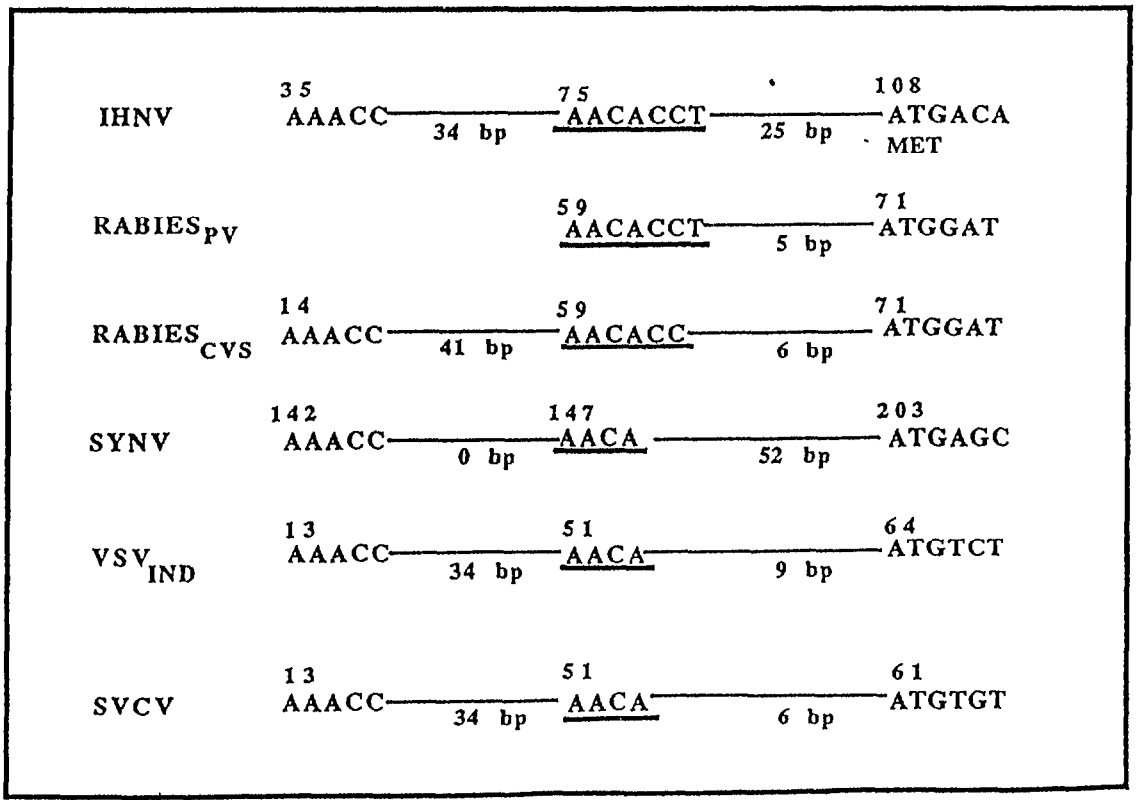

IHNV
35
AAACC
34 bp
75
AACACCT
25 bp
108
ATGACA
MET
RABIES$_{PV}$
59
AACACCT
5 bp
71
ATGGAT
RABIES$_{CVS}$
14
AAACC
41 bp
59
AACACC
6 bp
71
ATGGAT
SYNV
142
AAACC
0 bp
147
AACA
52 bp
203
ATGAGC
VSV$_{IND}$
13
AAACC
34 bp
51
AACA
9 bp
64
ATGTCT
SVCV
13
AAACC
34 bp
51
AACA
6 bp
61
ATGTGT

TABLE 1

COMPARISON OF N PROTEIN HOMOLOGY

Virus	% Homology (No. of identical amino acids)				
	VSV-IND	VSV-NJ	RAB-PV	SYNV	IHNV
VSV-IND	X	68.72	21.43	13.94	12.94
VSV-NJ		X	22.62	13.70	10.66
RAB-PV			X	8.90	9.98
SYNV				X	12.07
IHNV					X

Note. The percentage homologous amino acids was determined by progressive sequence alignment with a gap penalty of 8 (*15*).

trarily designated position 1 is shown in Fig. 1A. An open reading frame beginning with an ATG codon at position 107 and terminating at position 1346 is shown with the predicted amino acid sequence. This frame potentially encodes a protein of 413 amino acids with a calculated molecular weight of 45,600 Da. This is slightly larger than the size estimated by migration of the IHNV N protein in SDS–polyacrylamide gels to be 40,500 Da for this Round Butte isolate of IHNV (*14*). However, the difference in size may be accounted for by phosphorylation of the N protein (*6*). No other reading frame has a potential for encoding more than 75 amino acid residues.

The determination of the nucleotide sequence of the mRNA encoding the IHNV N protein made it possible for us to examine the extent of relatedness between IHNV, VSV New Jersey and Indiana, rabies virus, and sonchus yellow net virus (SYNV). This analysis showed no significant homology at the nucleotide level between the N genes of IHNV and VSV, rabies virus, or SYNV. These findings were not unexpected because there was no hybridization between IHNV N gene probes and VSV N mRNA on Northern blots (data not shown). In contrast, amino acid sequence comparisons of the N proteins by progressive similarity alignments (*15*) indicate that there are amino acid sequences that are conserved among these rhabdoviruses. For the N protein of IHNV, the range in amino acid identities was approximately 10–13% (Table 1 and Fig. 2). This is remarkably different from the glycoproteins where the range was 19–20% (*11*). These results indicate that these rhabdoviral N genes shared a common ancestor but the SYNV and IHNV genes diverged considerably earlier from the rabies and VSV genes. The conservation of sequences observed for the glycoproteins may indicate constraints on amino acid substitutions imposed by the glycoproteins function in virus infection.

An analysis of the relative hydropathicity of the N proteins was generated based on values published for each amino acid by Kyte and Doolittle (*16*). The most consistent finding was that of a very hydrophilic region at the carboxy terminus of all N proteins (data not shown). For IHNV, this hydrophilic tail contained highly charged basic amino acids; 12 of the last 57 (mol% = 21%) amino acid residues were arginine. There were no lysine residues in this region and the basic/acidic amino acid ratio was 2.4, similar to that of the arginine-rich histones. These observations make it tempting to speculate that this region is involved in the binding of the N protein to viral RNA.

The IHNV N gene was cloned from both genomic and messenger RNAs and sequenced. The exact 5′ end of the mRNA molecule was not determined, leaving the number of nucleotides at the 5′ end of the mRNA ambiguous. However, a sequence of 107 bp at the 5′ end of the N gene before the first ATG start site was detected in both an mRNA and a genomic RNA cDNA clone. With the exception of SYNV, a plant rhabdovirus which has a 5′ noncoding region of 56 bases on its N gene mRNA (*17*), other rhabdovirus mRNA sequences have only 10–30 bases before the translation start site on the mRNA (*18*). The sequence of the IHNV G mRNA indicated that there was a minimum of 48 bases of noncoding sequence at the 5′ end of the G mRNA (*11*). Thus, IHNV mRNAs appear to have longer 5′ noncoding regions than previously described rhabdoviruses. This observation raised interesting speculation concerning the molecular biology of the IHN virus.

Leader RNAs are transcribed from the 3′ end of the genome, immediately upstream of the N gene, and are thought to serve an important role in the transcription and replication of rhabdoviruses (*19*). Leader RNAs of 47 to 50 bases have been detected in VSV (*20*), rabies virus (*21*), and SVCV (*22*); and SYNV has a leader RNA

FIG. 2. Comparison of N protein sequences and 5′ noncoding region of the genomes of IHNV, VSV, rabies virus, and SYNV. (A) The IHNV N protein was compared with the other rhabdovirus N proteins (*17, 27, 26*) by progressive multiple alignments as described (*15*). Only those amino acids which are identical between IHNV and the other rhabdovirus N genes have been boxed. Close examination of the alignment will show that there are many other identical amino acids between these N gene sequences. (B) A comparison of the 5′ noncoding regions of genomic RNA for IHNV, rabies virus (*26*), VSV (*21, 28, 29*), SYNV (*17*), and SVCV (*22*). The leader sequences are shown in the (+) sense as cDNA and the position from the start of the viral genome is shown above each sequence. Bases marked by underlining indicate the consensus sequence for the initiation of the N mRNA synthesis. That position is hypothetical for IHNV.

of 147 bases (*23*). All leader RNAs described were transcribed separately from the viral mRNAs, and no precursor forms were detected in which leader RNA was attached to a mRNA. Polycistronic mRNAs have been detected during VSV replication; however, leader RNA probes were not used in these studies to determine if leader RNA was attached to the polycistronic mRNAs (*24*). Although the 5′ noncoding region of the IHNV N mRNA shares five (3′-UUUGG-5′) (positions 35–39) of the consensus hexanucleotide 3′-UUUGGU-5′ found in rhabdovirus leader RNAs (*25, 21*), it is unclear whether this sequence is an invariant characteristic of rhabdoviral leader RNAs (Fig. 2B). This homology is not found in the Pasteur strain of rabies (*26*). The exceptionally long 5′ noncoding region of the IHNV N gene and electron micrographs of R-loops formed between IHNV genomic RNA and plasmids containing cloned IHNV N cDNA sequences (*8*) suggest that the equivalent of an IHNV leader sequence may be transcribed in IHNV as part of the N mRNA. This model is only speculative at this point and direct RNA sequencing of the genome is under way to determine the 3′-terminal base sequences. Leader RNA detection experiments will also help to clarify the existence of the putative leader RNA and the mechanism by which it is transcribed.

ACKNOWLEDGMENTS

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MANUSCRIPT # 8

Glycoprotein from Infectious Hematopoietic Necrosis Virus (IHNV) Induces Protective Immunity against Five IHNV Types

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Glycoprotein from Infectious Hematopoietic Necrosis Virus (IHNV) Induces Protective Immunity against Five IHNV Types[1]

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Abstract.—Infectious hematopoietic necrosis virus (IHNV) is a fish pathogen that kills young salmon and trout. Outbreaks of the disease among hatchery-reared fish are a problem in the northwestern USA from northern California to Alaska. At least five biochemical types and several strains of differing host specificity of IHNV exist. Any vaccine developed to immunize fish must be able to elicit a response that will neutralize all strains of IHNV. This report shows that a single type of IHNV can induce a protective immune response in vivo to the five biochemical types of IHNV and indicates that, of the IHNV isolates examined, there is at least one common major neutralization epitope. Therefore, a vaccine developed against this common neutralization epitope will provide cross-protective immunity against these IHNV variants.

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that infects salmon and trout. It produces an acute disease resulting from the destruction of the hematopoietic tissue in the kidneys and may lead to the loss of an entire fish hatchery population (Pilcher and Fryer 1980). The only effective means to control this disease has been the complete destruction of stocks of infected fish and hatchery sterilization. These control methods are expensive and, in some cases, lead to the destruction of valuable fish stocks. Therefore, an effort was made to develop a vaccine for IHNV.

Previous studies have indicated that protective immunity to IHNV was induced in sockeye salmon *Oncorhynchus nerka* with a strain of IHNV that had been passed more than 40 times on STE-137 cells of steelhead *Oncorhynchus mykiss* (formerly *Salmo gairdneri*: Fryer et al. 1976; Tebbit 1976). However, this attenuated strain of IHNV was actually lethal to approximately 20% of immunized young rainbow trout *O. mykiss* (J. R. Winton, National Fisheries Research Center, unpublished). To avoid some of the problems attendant with modified live virus vaccines, we undertook development of a subunit vaccine against IHNV.

Our first objective was to identify the viral protein responsible for the induction of protective immunity in fish. For the rhabdoviruses that cause vesicular stomatitis and rabies, the viral glycoprotein is the only viral antigen that induces neutralizing antibody and protective immunity (Kelley et al. 1972; Cox et al. 1977; Wiktor et al. 1984). The IHNV glycoprotein has also been shown to be the sole viral antigen responsible for inducing protective immunity in fish (Engelking and Leong 1989). However, the ability of this IHNV glycoprotein to induce a cross-protective immunity to other virus isolates had not been determined. Thus, the glycoprotein of IHNV from a type-1 strain was tested in vivo to determine its capacity to induce protective immunity to five different biochemically defined types of IHNV (Hsu et al. 1986). We used purified IHNV glycoprotein from the Round Butte type-1 (RB1) strain to induce cross-protective immunity in salmonid fry to five biochemical types of IHNV. This IHNV isolate has been extensively characterized biologically and biochemically (Mulcahy et al. 1984; Kurath et al. 1985; Koener et al. 1987). Induction of immunity occurred after immersion of fish as small as 0.4 g in a solution of purified glycoprotein, and protective immunity was produced for at least 30 d.

Methods

Cells and virus.—Chinook salmon *Oncorhynchus tshawytscha* embryo cells (CHSE-214) were grown in RPMI-1640 medium supplemented with fetal calf serum (5%), penicillin (100 international units [IU]/mL), and streptomycin (100 μg/mL), as previously described by Engelking and Leong (1981).

Virus assays were performed on confluent CHSE-214 cell monolayers grown in 24-well tis-

[1] Oregon Agricultural Experiment Station Technical Papers 8595 and 8596.

sue culture plates (Falcon). Samples from infected fish were prepared as described by Amos (1985), sterilized by filtration (0.2-μm-pore acrodisc, Gelman), and diluted in minimal essential medium (MEM) without fetal calf serum. Duplicate samples of 0.1 mL from each dilution were placed on monolayers in individual wells and allowed to adsorb for 60 min. Sample inocula were removed from the wells after adsorption, and 1.0 mL of MEM growth medium was added to each well.

Five IHNV isolates were used, each representing a different biochemical type. The Round Butte type-1 (RB1) and the Elk River type-3 (ER) strains of IHNV were obtained from W. Groberg, Oregon Department of Fish and Wildlife. The Hagerman Valley type-2 (HA1) strain was obtained from N. Wood, Rangen Research Laboratories, Idaho. The Coleman National Fish Hatchery type-4 (CO2) strain and Cedar River type-5 (CD2) strain were obtained from D. Mulcahy, National Fisheries Research Center, Seattle, Washington. The viral glycoprotein was isolated from the Round Butte strain of IHNV after extensive purification of the virus by isopycnic and velocity sedimentation. All virus strains were prepared by growing the virus at a multiplicity of infection (MOI) of 0.001 plaque-forming units (PFU) per cell on CHSE-214 cells, as previously described by Engelking and Leong (1981).

The virus used for challenges in the immunization trials was prepared from a stock of virus that had undergone no more than three passes in tissue culture after isolation from infected fish.

Virus purification.—Virus was propagated in CHSE-214 cells grown in 175-cm^2 plastic tissue culture flasks (Falcon). The cell monolayers were infected at an MOI of 0.001 PFU/cell and incubated at 16°C for 7 d or until the monolayers showed a complete cytopathic effect (CPE). The supernatant fluid was harvested and cell debris was removed by centrifugation at 3,000 × gravity for 10 min at 4°C in a Sorvall HS-4 rotor. The virus lysate was centrifuged onto a 0.3-mL pad of 100% glycerol with a Beckman SW28 rotor for 60 min at 90,000 × gravity. The virus pellet was resuspended in 0.01-M tris-HCl buffer at pH 7.5. Ten milliliters of this virus suspension were centrifuged through a discontinuous gradient composed of 7 mL of 50% sucrose–0.01-M tris (pH 7.5), 10 mL of 35% sucrose–0.01-M tris (pH 7.5), and 10 mL 20% sucrose–0.01-M tris (pH 7.5) for 90 min at 90,000 × gravity in a SW28 rotor. The virus band was collected from the interface between the 20% and 35% sucrose layers. This band was concentrated by centrifugation in 0.01-M tris (pH 7.2) at 90,000 × gravity for 1 h. The pellet was resuspended in 0.01-M tris at pH 7.5 and banded in a continuous gradient of 5–30% sucrose–0.01-M tris at pH 7.5 in a SW41 rotor at 49,000 × gravity for 30 min. The virus band was collected and concentrated by centrifugation in 0.01-M tris at pH 7.5 in a SW41 rotor for 1 h at 100,000 × gravity and 4°C. The resulting virus pellet was resuspended in 0.5 mL 0.01-M tris at pH 7.5 and stored at −70°C.

Purification of the viral glycoprotein.—The IHNV glycoprotein was purified as previously described by McAllister and Wagner (1975). Briefly, purified IHNV in 0.01-M tris at pH 7.4 was incubated in 1% Triton X-100 at 25°C for 60 min to selectively solubilize the glycoprotein. The soluble and particulate fractions were separated by centrifugation at 140,000 × gravity for 60 min at 4°C in a SW50.1 rotor. The glycoprotein was found in the supernatant fluid free of other viral proteins. The other viral proteins were in the pelleted particulate fraction. The Triton X-100 in the glycoprotein preparation was then removed by batch elution with SM-2 beads (Bio-Rad). A homogeneous purified glycoprotein preparation was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). We used a 10% polyacrylamide gel with a 4.5% stacker, as described by Laemmli (1970). Protein bands were visualized by staining the gel with Coomassie brilliant blue or silver nitrate, as described by Allen (1980).

Immunization of fish.—Kokanee (lacustrine sockeye salmon) and rainbow trout used in these studies were obtained from Oregon Department of Fish and Wildlife hatcheries through Richard Holt, Oregon State Fish Pathologist. The fry, 1–2 months old and weighing approximately 0.3 g/fish, were obtained at least 1 week before immunization and acclimated to the well water at the Fish Disease Laboratory in Corvallis, Oregon. The fry were maintained on BioDiet (Bio-Products, Warrenton, Oregon) in well water flowing at 1.9 L/min at 12°C. When the fish weighed 0.4 g, they were vaccinated by direct immersion.

Immersion immunization was performed on 100 unanesthetized fish in a beaker containing 4 mL of purified glycoprotein (40–60 μg/mL) in 0.01-M tris at pH 7.6. The fish were exposed to the protein solution for 1–2 min and then released into a holding tank containing running water at 12°C. Fish were held for 30 d before being challenged by virus.

Virus challenge.—Challenges were made with lots of 25 vaccinated and 25 unvaccinated control fish for each virus dilution (Johnson et al. 1982a, 1982b). The fish were exposed to serial $\log_{10}$ virus dilutions in 1 L of water. These dilutions represented approximately 1×10^4 and 1×10^3 of the tissue culture infectious dose that resulted in 50% mortality (TCID50) per mL of water. The actual titer was determined for each experiment by the method of Reed and Muench (1938). The fish were held in the virus-containing water for 18 h and then returned to holding tanks. Dead fish were removed daily from each tank to be weighed and processed.

Isolation of IHNV from infected fish.—Dead fish were processed immediately for virus isolation. The fish were weighed and diluted 1:10 (weight: volume) with Hanks' balanced salt solution (HBSS), and then homogenized in a Stomacher processor (Tekmar). The resulting suspension was clarified by centrifugation, and the supernatant solution was treated (1:5 dilution) with antibiotics (penicillin, 1,000 IU/mL; streptomycin, 1,000 μg/mL; Fungazone, 500 IU/mL; and gentamicin, 0.25 μg/mL) in HBSS overnight at 4°C. The next day the fluid was inoculated directly onto CHSE-214 cells in 24-well plates, as previously described by Amos (1985). The cells were observed daily for cytopathic effects for 2 weeks. For those samples in which CPE was questionable, the tissue culture fluid from the sample well was removed and reinoculated onto CHSE-214 cells and subsequently labeled with ^{35}S-methionine, as described by Hsu and Leong (1985).

Only those fish from which IHNV was isolated were considered in these studies. The percent mortality was determined for each group, and the relative percent survival (RPS; Johnson et al. 1982b) was calculated for each group of vaccinated fish as

$$\text{RPS} = \left[1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{mortality of control fish}}\right] \times 100;$$

when mortality of vaccinated fish is greater than that of control fish, RPS = 0.

Statistical analysis.—The data were analyzed by logit regression. The number of fish that died from IHNV infection was assumed to be a binomial random variable with the probability of death, p, depending on the various factors under investigation.

Results

Challenge of Fish with Homologous IHNV Strain

Experiments with rainbow trout and kokanee fry previously showed that immunization with purified glycoprotein from the Round Butte strain of IHNV does elicit a protective response to subsequent challenge with the homologous strain of IHNV (Table 1). The protection of kokanee against IHNV challenge from homologous virus occurred in glycoprotein-vaccinated fish. In four experiments, an RPS of 83 was achieved at typical environmental IHNV levels (3.8×10^3 TCID50/mL). At a concentration of IHNV at which almost 90% of unvaccinated fish died (3.8×10^6 TCID50/mL), an RPS of 47 was achieved and only 48% of the vaccinated fish died (Table 1). Similar results were obtained with rainbow trout: at levels of IHNV that naturally occur during epizootics, an RPS of 60 was achieved in glycoprotein-immunized fish (Table 1); the difference in survival between immunized and unimmunized fish was highly significant ($P < 0.01$). In general, better protection was afforded at lower concentrations of virus, as indicated by the RPS values. These lower amounts of virus were representative of natural levels that fish encounter before epizootic outbreaks (Mulcahy et al. 1983).

Challenge of Fish with Heterologous IHNV Strain

The heterologous virus isolates used for challenge were biochemically grouped on the basis of differences in the apparent mass of their respective nucleocapsid protein or glycoprotein (Tables 2, 3). The type-1 biochemical group was represented by an isolate of IHNV from Oregon. This group has a nucleocapsid protein with a mass of 40.5 kilodaltons (kDa) and a glycoprotein of 67 kDa. The other isolates were from different geographic regions and differed in the apparent mass of their nucleocapsid proteins (Table 2). The type-4 isolate from Coleman National Fish Hatchery also differed in the apparent mass of the glycoprotein; the CO2 isolate had a glycoprotein that appeared to be 3–3.5 kDa greater than that of the other IHNV isolates when comparisons were made by gel migration (Table 3; Figure 1). Fish were immunized with glycoprotein isolated from type-1 IHNV (RB1) and challenged with one of the different IHNV types.

The glycoprotein from RB1 (type 1) induced

TABLE 1.—Survival, after challenge with type-1 IHNV, of kokanee and rainbow trout fry immunized by exposure to glycoprotein of type-1 IHNV strain RB1.

IHNV titer used for challenge (TCID50/mL)[a]	Control fish			Immunized fish			
	N	Specific loss[b]	Percent mortality	*N*	Specific loss[b]	Percent mortality	Relative percent survival[c]
			Kokanee[d]				
3.8×10^6	75	64	89	75	36	48	47
3.8×10^5	107	86	80	100	40	40	50
3.8×10^4	112	66	59	100	18	18	70
3.8×10^3	105	31	30	100	5	5	83
3.8×10^2	30	5	17	27	3	11	35
			Rainbow trout[e]				
1.1×10^6	50	36	72	50	19	38	47
1.1×10^5	50	35	70	50	14	28	60
1.1×10^4	50	6	12	50	4	8	34
1.1×10^3	50	1	2	46	3	6	0

[a] TCID50 is the tissue culture infective dose that causes a cytopathic effect in 50% of the test cultures.
[b] Specific loss is the number of fish that died as a result of the challenge.
[c] Relative percent survival (RPS) is 100(1 − [% mortality of vaccinated fish/% mortality of control fish]); RPS = 0 if % mortality of vaccinated fish equals or exceeds that of controls.
[d] Combined results of four trials.
[e] Combined results of two trials.

protection against all the other types of IHNV tested (Table 4). Immunized rainbow trout achieved an RPS of 70 at a dose of type-2 IHNV that was lethal to more than 90% of unimmunized control fish (Table 4). At levels of virus at least 10-fold greater than naturally occurring concentrations, an RPS of 80 was achieved in immunized rainbow trout exposed to type-3 IHNV (Table 4). With challenge titers of 4.6–5.0 × 10^3 TCID50/mL, the RPS was 84 for the type-4 IHNV test and 63 for the type-5 test. Significant protection did not occur ($P > 0.05$) only in the first experiment with the type-2 IHNV isolate from Hagerman Valley; however, the sample size was statistically too small for that test. A significant level of protection was afforded by vaccination with the RB1 glycoprotein against type-2 IHNV in the second test. The most effective cross-protective immunity was induced against chinook salmon IHNV isolates from Elk River and the Coleman Hatchery (Table 3).

LD50 Comparison of Challenge with Heterologous IHNV

A comparison was made of the amount of virus required to kill 50% of the infected fish (LD50) in vaccinated and control groups (Table 5). The LD50 was estimated for groups that did not reach 50% mortality. The LD50 for the mock-immunized control groups ranged from 2.3 × 10^4 to 4.2 × 10^2 TCID50/mL of IHN challenge virus. The high dose of 2.3 × 10^4 for IHNV from Elk River required to produce an LD50 in rainbow trout was possibly due to this isolate's adaptation to chinook

TABLE 2.—Description of variants of infectious hematopoietic necrosis virus.

Variant	Biochemical type[a]	Host species: geographic origin	Tissue source	Date of isolation
RB1	1	Summer steelhead: Round Butte Hatchery, Oregon	Ovarian fluid	1975
HA1	2	Rainbow trout: Hagerman Valley, Idaho	Adult tissue	1978
ER	3	Fall chinook salmon: Elk River, Oregon	Fry tissue	1979
CO2	4	Chinook salmon: Coleman National Fish Hatchery, California	Alevin tissue	1980
CD2	5	Fall chinook salmon: Cedar River, Washington	Ovarian fluid	1981

[a] Biochemical types are described in Table 3.

TABLE 3.—Biochemical types of infectious hematopoietic necrosis virus defined by the mass of constituent proteins.[a]

Type	Mass (in kilodaltons) of	
	Nucleocapsid protein	Glycoprotein
1	40.5	67
2	42.8	67
3	43.25	67
4	40.5–41.0	70
5	41.0–44.0[b]	67

[a] Adapted from Hsu et al. (1986).
[b] This grouping is less well defined and has nucleocapsid proteins of different masses.

FIGURE 1.—Comparison of five types of IHNV by sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis. The identified proteins (coded at right) are L protein (L: RNA polymerase) of molecular mass 150 kilodaltons (kDa), glycoprotein (G), nucleocapsid protein (N), M_1, and M_2. **(Lane A)** Purified IHNV from Round Butte Hatchery, Oregon (type 1): G = 67 kDa; N = 40.5 kDa; M_1 = 25 kDa; M_2 = 22.5 kDa. **(Lane B)** Purified IHNV from Coleman National Fish Hatchery, California (type 4): G = 70 kDa; N = 40.5–41 kDa. **(Lane C)** Purified IHNV from Cedar River, Washington (type 5): N = 43 kDa. **(Lane D)** Molecular mass markers: phosphorylase B, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa. **(Lane E)** IHNV from Hagerman Valley, Idaho (type 2): N = 42.8 kDa. **(Lane F)** IHNV from Elk River, Oregon (type 3): N = 43.25 kDa.

TABLE 4.—Survival, after challenge with IHNV types 2–5, of kokanee and rainbow trout fry immunized by exposure to glycoprotein of IHNV type 1.

Challenge virus		
Type	Titer (TCID50/mL)[a]	Relative percent survival[a]
	Kokanee	
2	2.0×10^4	0
	2.0×10^3	20
	Rainbow trout	
2	4.2×10^4	70
	4.2×10^3	53
3	1.0×10^5	80
	1.0×10^4	100
	1.0×10^3	100
4	5.0×10^3	84
	5.0×10^2	100
5	4.6×10^3	63
	4.6×10^2	70

[a] See Table 1 for definitions.

salmon (Chen 1984). The other LD50 values were in the range that had been reported previously for rainbow trout (Amend and Nelson 1977; Chen 1984). Fish immunized by immersion required a dose of approximately 10–200 times more virus than control fish to produce 50% mortality (Table 5).

Discussion

The envelope glycoprotein of IHNV was purified and shown to induce protective immunity in fish against challenge with either homologous or heterologous types of virulent IHN virus. Thus, IHNV was similar to rabies and vesicular sto-

TABLE 5.—Doses (LD50) of IHNV types that killed 50% of immunized and unimmunized (control) fish in challenge experiments. Immunized kokanee and rainbow trout fry were immersed in a bath containing purified glycoprotein from IHNV type-1 strain RB1. The LD50 is given as a multiple of the tissue culture infective dose (TCID50).

Challenge virus		LD50 (as TCID50/mL)		LD50 ratio (I:C)
Isolate	Type	Immunized fish (I)	Control fish (C)	
		Kokanee		
RB1	1	5.8×10^4	1.8×10^3	32
HA1	2	6.9×10^3	2.1×10^3	3
		Rainbow trout		
RB1	1	8.4×10^4	3.0×10^3	28
HA1	2	4.2×10^3	4.2×10^2	10
ER	3	$>2.3\times10^5$	2.3×10^3	>100
CO2	4	$>5.0\times10^5$	5.0×10^3	>100
CD2	5	4.6×10^5	2.4×10^3	>190

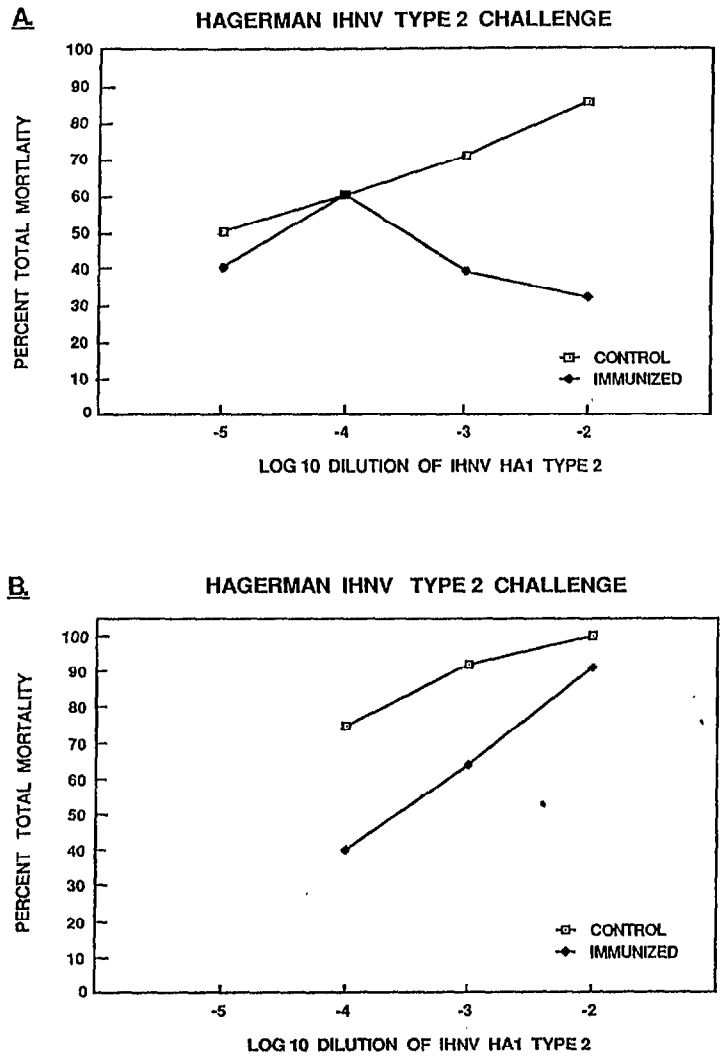

FIGURE 2.—Percent mortality of immunized and unimmunized (control) kokanee (A) and rainbow trout (B) after challenge with different $\log_{10}$ dilutions of IHNV type-2 strain HA1. Fish were immunized in a bath containing glycoprotein from IHNV type-1 strain RB1.

matitis viruses, both mammalian rhabdoviruses with envelope glycoproteins that are solely responsible for inducing neutralizing antibody and protective immunity in the host (Kelley et al. 1972; Cox et al. 1977; Wiktor et al. 1984).

Fish can be effectively vaccinated against concentrations of IHNV normally found in the environment before epizootics (10^3–10^4 TCID50/mL) by immersion in a solution of purified glycoprotein. Immunized fish had RPS values of 60 or greater at these concentrations of IHNV. This level of protection is acceptable for hatchery fish (Johnson et al. 1982b). At concentrations of IHNV that are typical of epizootic conditions (10^5–10^6 TCID50/mL), some protection is still afforded by glycoprotein immunization (Table 1; Figures 2–4).

Type-1 IHNV RB1 was highly virulent in kokanee and produced greater mortality of this species at every concentration than it did of rainbow trout (Table 1). Even at a low dose (10^2 TCID50/mL) of IHNV RB1, 17% of the control-challenged kokanee died, whereas only 2% of the rainbow trout died at 10^3 TCID50/mL. Approximately twice the amount of IHNV was required for rainbow trout than for the kokanee to produce

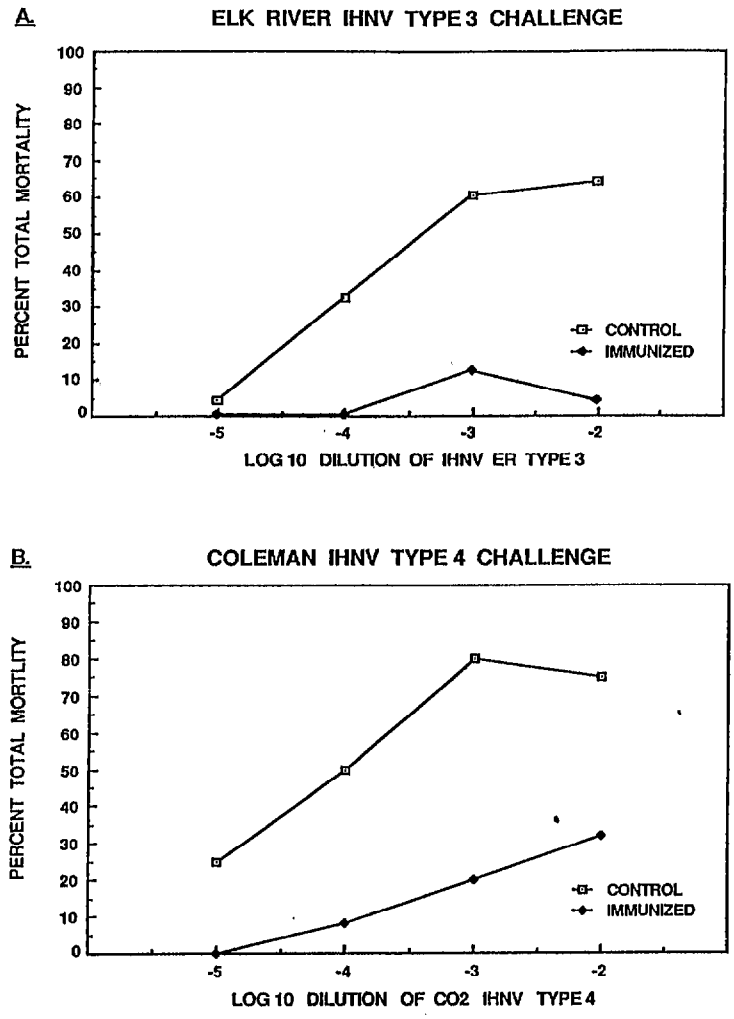

FIGURE 3.—Percent mortality of immunized and unimmunized (control) rainbow trout after challenge with different $\log_{10}$ dilutions of IHNV type-3 strain ER (A) or type-4 strain CO2 (B). Fish were immunized in a bath containing glycoprotein from IHNV type-1 strain RB1.

an LD50 (Table 5). It was possible that the virulence exhibited by type-1 IHNV for kokanee was an adaptation that permitted that virus type to grow more rapidly in that fish species. The vaccination of rainbow trout and kokanee with purified glycoprotein from type-1 IHNV RB1 induced a more effective protective response against a challenge of IHNV of 10^3–10^4 TCID50/mL than against higher concentrations of IHNV (Table 1). At IHNV concentrations of 10^5–10^6 TCID50/mL, it was probable that the virus had mounted a pathogenic infection of sufficient rapidity to partially overwhelm the fish's immune response. Similar effects have been observed in vaccination trials for foot-and-mouth disease (Kleid et al. 1981). However, these large concentrations of virus do not naturally occur before IHNV epizootics (Mulcahy et al. 1983). In spite of the greater mortality at higher concentrations of IHNV, the vaccination still protects, albeit to a lesser extent (RPS < 60).

For the four cross-protection experiments, an LD50 was achieved in the control groups at the naturally occurring concentrations of IHNV, except in the case of the Elk River type-3 IHNV. The rainbow trout host fish used in this trial were apparently less susceptible to this virus strain than the natural chinook salmon host species. Thus, the rainbow trout were also challenged with an additional 10-fold higher dose of ER IHNV (10^5

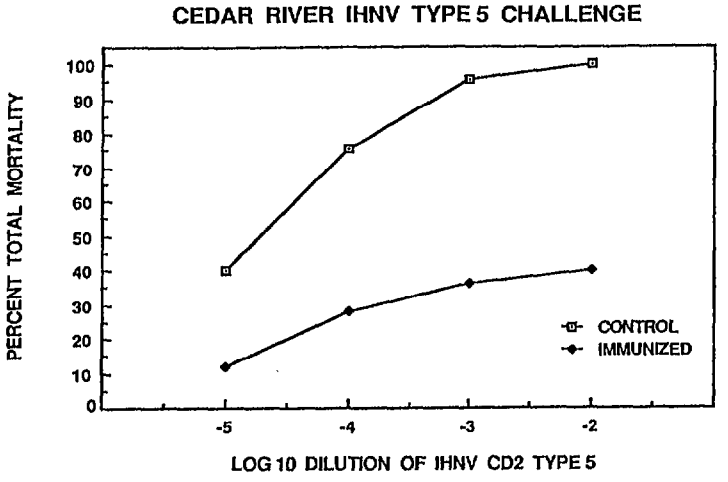

FIGURE 4.—Percent mortality of immunized and unimmunized (control) rainbow trout after challenge with different $\log_{10}$ dilutions of IHNV type-5 strain CD2. Fish were immunized in a bath containing glycoprotein from IHNV type-1 strain RB1.

TCID50/mL) (Table 4; Figure 3). At LD50 concentrations of IHNV, the immunized fish were well protected in all cases (Table 3). At greater virus concentrations that caused about 100% mortality in the control fish groups, significant reductions in mortality of 50–90% ($P < 0.05$) still occurred in the vaccinated fish (Figures 2–4). Statistically significant protection was afforded by glycoprotein vaccination in all cross-challenges. The best protection from cross-strain challenge with IHNV was achieved with the Coleman Hatchery (CO2) and Elk River (ER) isolates. Values of RPS of 80–100 were determined for the immunized fish in these trials, which indicated complete protection (Table 4).

Good protection was achieved in the other two IHNV cross-strain challenge groups with the Hagerman Valley (HA1) and Cedar River (CD2) isolates. The low RPS values found in the first experiment with IHNV HA1 appeared to be the result of experimental variation from the small number in the sample (Table 1). If those results were disregarded, RPS values from 53 to 70 were determined for the immunized groups (Table 4). Again, this would be considered effective immunity, as described by Johnson et al. (1982b), who set an RPS value of 60 for adequate protection with bacterial vaccines. The difference in susceptibility of some salmonids to certain strains of IHNV may account in part for the lower RPS values. There was greater mortality in the control groups of these two trials than in the other cross-protection trials (Table 4). If this reflects increased virulence of these IHNV strains, a greater immunogenic mass or booster immunization may be required to produce adequate protection.

The level of protection can also be assessed by determining the amount of virus required to produce an LD50 in immunized groups as compared to that required for control groups. This type of comparison also showed that vaccination was effective (Table 5). An infectious dose of IHNV approximately 10–200 times that of the control groups needed to be administered to produce an LD50 in the groups immunized by immersion. The required dose may be even greater because, in some cases, an LD50 was not obtained in the immunized groups and therefore was estimated (Table 5). The level of IHNV protection was similar to that seen with the homologous virus, RB1, which required 28–32 times more virus to produce an LD50 in the immunized fish. This dramatic protection suggested that IHNV epizootics would not occur in glycoprotein-immunized fish. To kill a significant number of the immunized fish and start a disease outbreak, 100 times more IHNV than the amount existing naturally before epizootics would be required.

Although these experiments were carried out for only 1 month, the cumulative mortality data suggested that the duration of immunity should last longer than that period of time. These studies did not include any experiments that accurately measured the duration of immunity because salmon and trout are refractory to the pathogenic effects of IHNV infection after 6 months (Pilcher and

Fryer 1980). A method to detect neutralizing antibodies in fish by other means than virus challenge is being developed for these studies. The enzyme-linked immunosorbent assay and other immunological methods employing goat anti-trout antiserum are being tested. In addition, the effects of booster doses and adjuvants on the duration of immunity are being tested.

These experiments clearly indicated that the viral glycoprotein alone, devoid of any other component of the virus particle, is able to confer immunity similar to that of other rhabdoviruses (Wiktor et al. 1984). These experiments also showed that the IHNV variants (types 1–5) have at least one conserved neutralization epitope. This indicated that a vaccine developed against the glycoprotein of one strain will elicit a protective response in fish to all IHNV variants. Therefore, the production of a recombinant vaccine based on a single type of IHNV is possible. Fish in any geographic area may be protected from endemic as well as exotic IHNV types, and the development of a more costly polyvalent vaccine may not be necessary.

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MANUSCRIPT # 9

The Glycoprotein of Infectious Hematopoietic Necrosis Virus Elicits Neutralizing Antibody and Protective Responses

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The glycoprotein of infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses

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Summary

Infectious hematopoietic necrosis virus (IHNV) is the etiologic agent of a disease that causes massive mortalities among hatchery reared salmon and trout fry. The severity of the economic losses resulting from this disease to the aquaculture industry has made it important to develop a means of preventing these disease outbreaks. Thus, the development of an IHNV vaccine was begun. In this study the glycoprotein G alone was shown to be the only viral protein necessary to elicit neutralizing antibodies. Furthermore, when susceptible salmonid fish were immunized with purified G protein, these fish survived subsequent viral challenge. Antiserum prepared to enzymatically deglycosylated virus was found to contain virus neutralization activity and to react with glycosylated G protein as well. This result indicated that some G protein epitopes were not carbohydrate in nature nor dependent on carbohydrates for their immunogenic conformation. Thus the viral G protein in its nonglycosylated form might be sufficient for a subunit vaccine for IHNV and prokaryotic expression of this viral gene in vaccine development was feasible.

Infectious hematopoietic necrosis virus; Vaccine; Glycoprotein; Rhabdovirus neutralization

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Introduction

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus with structural similarity to vesicular stomatitis virus (VSV) and rabies virus; its five virion proteins resemble that of rabies virus (McAllister and Wagner, 1975). The viral genome is unique among rhabdoviruses, however, in that it encodes and expresses a sixth viral protein found only in infected cell lysates (Kurath and Leong, 1985). IHNV is a major viral pathogen of salmonid fish in which the hematopoietic tissue of the anterior kidneys and spleen are destroyed by the virus (Pilcher and Fryer, 1980). The numbers of fish killed by the infection often approach 100% in hatcheries and thus, the development of an IHNV vaccine was undertaken.

To develop a vaccine for IHNV, the virion protein(s) responsible for inducing a protective immunity in fish had to be identified. For VSV and rabies virus, the surface glycoprotein G of the virus has been shown to be responsible for eliciting both neutralizing antibody and protective immune responses in mammalian hosts (Kelley et al., 1972; Cox et al., 1977; Wiktor et al., 1984). The IHN virion also contained a surface glycoprotein whose gene had been cloned and whose nucleic acid sequence had been determined (Koener et al., 1987). A comparison of the deduced amino acid sequence of the IHNV glycoprotein with that of the cloned glycoprotein genes of VSV and rabies virus indicated some conservation of sequence and, more importantly, structural similarity. These similarities suggested that the IHNV glycoprotein might also induce a protective immunity in fish. Previous studies had shown that passive immunity to IHNV could be transferred with serum from fish that survived an IHNV infection and that this serum contained virus neutralizing activity (Amend and Smith, 1975). A high passage attenuated IHNV strain was protective in fish for a period of at least 100 days (Fryer et al., 1976).

The antigenicity of the IHNV glycoprotein was examined for both purified virus and purified viral glycoprotein. The virus neutralizing capacity of the antisera was compared. Included in this study was an analysis of the immune response to N, the nucleocapsid phosphoprotein; M1, a phosphorylated matrix protein; and M2, the other virion matrix protein. The G, N, M1 and M2 proteins comprise 95% of the total amount of virion protein (McAllister and Wagner, 1975; Hill, 1975). After identifying the glycoprotein as the only viral protein capable of evoking neutralizing antibody in rabbits, the purified G protein was tested as a vaccine for IHNV in salmon and rainbow trout. The purified G protein induced protective immunity in fish vaccinated by immersion or injection.

Materials and Methods

Cells and virus

The chinook salmon embryo cell line (CHSE-214) was used for propagating IHNV as previously described (Engelking and Leong, 1981). The cells were grown as monolayers in RPMI-1640 medium (GIBCO Laboratories) supplemented with

5% fetal calf serum, penicillin 100 I.U./ml, and streptomycin 100 μg/ml. The IHNV used in this study was isolated in 1975 from an adult steelhead trout at the Round Butte Hatchery in Oregon. It has been referred to as RB1 (Hsu et al., 1986). The virus was propagated in fish cells at a multiplicity of infection of 0.001 at 15°C for 7 days (Engelking and Leong, 1981). The supernatant was then collected and centrifuged at $2500 \times g$ for 10 min at 4°C. The cell-free supernatant contained $0.5-1 \times 10^8$ $TCID_{50}$ (50% tissue culture infective doses) per ml as determined by the method of Reed and Muench (1938). Virus purification was performed on sucrose gradients as described (Leong et al., 1981).

Neutralization assays

Plaque assays were performed as described by Burke and Mulcahy (1980). Serial tenfold dilutions of virus were incubated with equal volumes of various dilutions of antisera at 15°C for 3 h on a rotating shaker. Duplicate wells in a six-well plate (Falcon) containing monolayer cultures of CHSE-214 cells were inoculated with 0.2 ml of each antiserum-virus mixture. After 1 h at 15°C, the infected cells were overlayered with 0.8% gum tragacanth (Fisher Scientific Co.) in minimal essential medium with 5% fetal calf serum. After seven to ten days at 15°C, the cells were fixed, stained, and the individual virus plaques were counted. The relative virus titers with and without antiserum treatment were determined and used to calculate the plaque reduction dilution end points.

Protein antigen purification

IHNV glycoprotein was purified from IHNV virion preparations by treatment with Triton X-100 to strip the glycoprotein from the virus surface. Centrifugation at $90,000 \times g$ in a SW50.1 rotor, for 60 min at 4°C was used to separate the solubilized glycoprotein from the remaining virus proteins. This procedure has been described for VSV (Kelley et al., 1972) and IHNV (McAllister and Wagner, 1975). A second purification method involved electroelution of the glycoprotein from gel after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with an Elutrap apparatus (Schleicher and Schuell). The third method involved separation of virion proteins by column chromatography. In this procedure, purified IHNV was solubilized in 1% SDS in 0.1 M ammonium bicarbonate buffer, pH 6.5. The viral proteins were separated on a gel filtration BioGel A-1.5 m matrix (BioRad) by size elution with 40 ml of 0.1 M ammonium bicarbonate buffer containing 0.1% SDS. Forty fractions of 1 ml each were taken and fractions nos. 16–18 contained the majority of the G protein with some contaminating N protein. This preparation of G protein was lyophilized, rehydrated and separated in the same manner on the BioGel A-1.5 m matrix. This preparation was lyophilized, rehydrated and shown to be homogeneous G protein as determined by SDS-PAGE and silver staining.

The IHNV proteins N, M1 and M2 were purified by electroelution from after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels. The

purity of each viral protein was verified by SDS-PAGE of the preparation and by silver staining as previously described (Hsu et al., 1986).

Deglycosylation of the IHNV glycoprotein

Purified IHN virions were deglycosylated enzymatically by the addition of an Endoglycosidase F: glycopeptidase F preparation from Boehringer Mannheim. About 1 mg of purified IHNV was reacted with 0.6 units of the enzyme mix under conditions that would remove all forms of N-linked carbohydrates, as described by the manufacturer. The incubation was for 24 h at 37 °C in 20 mM $KHPO_4$, pH 7.4, 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM 2-mercaptoethanol, 20 mM EDTA, 0.4% Triton X-100. The deglycosylated G protein was isolated by electroelution as above.

Antisera preparation

Antisera to purified virus or specific viral proteins were prepared in three-month-old New Zealand White male rabbits. A series of three immunization episodes were administered by intradermal injection at multiple sites. The first immunization dose contained either 250 μg of IHNV or 100 μg of specific protein antigen mixed 1 : 1 (v/v) with complete Freund's adjuvant. The second and third immunizations were given at two and four weeks later, respectively. These booster injections contained one half the amount of antigen used in the initial injection in a 1 : 1 (v/v) mixture with incomplete Freund's adjuvant. Blood samples were drawn from the animals at 7–10 days after the last booster injection. Those rabbits identified as hyperimmune by enzyme linked immunoassay (ELISA) were bled by cardiac puncture. The antisera were heat inactivated from 30 min at 56 °C and aliquots of the sera were stored at −70 °C.

Fish

Rainbow trout (*Oncorhynchus mykiss*) and kokanee salmon (*Oncorhynchus nerka*, Walbaum) fry were obtained through the Oregon Department of Fish and Wildlife. The virus challenge experiments were performed at the Oregon Department of Fish and Wildlife, Oregon State University Fish Disease Facility. The fish were immunized by immersion or anesthesized with benzocaine before immunization by intraperitoneal injection. After thirty days, lots of 25 fish were challenged with live, infectious IHNV by immersion in 1 l of water for 18–24 h containing different concentrations of the virus (Gilmore et al., 1988). Dead fish were collected daily and examined for visible signs of disease. Every dead fish was processed by standard methods (Amos, 1985) to determine whether the fish had died from an IHNV infection. The relative percent survival (RPS) was determined by the formula:

$$\text{RPS} = \left[1 - \frac{\%\ \text{loss immunized}}{\%\ \text{loss controls}}\right] \times 100$$

(Johnson et al., 1982a).

Protein analysis

The concentration of protein in different viral antigen preparations was determined by a dye binding method (BioRad). The proteins were analyzed in a discontinuous gel buffer system as described by Laemmli (1970) and detected by silver stain as previously described (Hsu et al., 1986).

RNA purification

Cellular RNA was isolated from infected cells and polyadenylated RNA was purified on oligo dT cellulose as described (Kurath and Leong, 1985).

In vitro translation and radioimmune precipitation

Translation of IHNV mRNA in vitro was performed with a rabbit reticulocyte lysate system (Promega) and the newly synthesized proteins were labelled with [^{35}S]methionine (DuPont, NEN). An aliquot of the translation reaction was mixed with antisera for 6 h at 4°C in the presence of protein A sepharose beads (Pharmacia). The beads containing bound viral protein-antibody complexes were centrifuged and washed three times with RIPA buffer (0.1% SDS, 0.2% Na deoxycholate, 0.5% NP40, 150 mM NaCl, 10 mM Tris, pH 7.4). After the final wash, the beads were resuspended in SDS sample buffer (Laemmli, 1970) and boiled for 2 min (Huang et al., 1986). Samples were analyzed by SDS-PAGE.

ELISA procedure

The antigen, purified IHNV, was diluted 1 : 1,000 to final concentration of 1 μg/ml in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) (TBS) and 200 ng were bound to each well of 96 well plates (Falcon, Pro Bind ELISA plates) by incubation overnight at 4°C. The plates were washed twice with TBS containing 0.5% Tween-30 (TBS-Tween) and then 200 μl of diluted antisera were added to each well. The plates were incubated for 1 h at room temperature and washed twice with TBS-Tween. Goat anti-rabbit horseradish peroxidase conjugated serum was added (1 : 1000 dilution) for 30 min at room temperature. The plates were then washed two times with TBS-Tween before the addition of 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) (Boehringer Mannheim) in 0.2% citrate with 0.1% (v/v) 30% hydrogen peroxide as the color development reagent. The plates were read at a wavelength of the 405 nm on a BioTek ELISA plate reader.

Immunoblot procedure

Purified IHNV was run on polyacrylamide gels (SDS-PAGE) as described above. The proteins from the gel were transferred to nitrocellulose filters by the Western blot technique as described by Towbin et al. (1979) in an electroblot apparatus. The blot was washed with 1 × TBS; then non-specific binding sites were blocked with a

3% gelatin solution in 1 × TBS. The blot was incubated at room temperature for 1 h with various dilutions of antisera. The dilution of antiserum used for different blots varied and was adjusted for maximum sensitivity for detection of the respective viral protein. It was then washed and incubated with 1 : 1000 dilution of goat antirabbit serum, conjugated with horseradish peroxidase (Sigma). After washing, the color development reagent 4-chloro-1-napthol (3 mg/ml) (Sigma) in 0.5 × TBS and methanol with 0.1% (v/v) 30% hydrogen was added. Rabbit antibody was detected in this manner.

Blocking neutralizing activity of antiserum directed against the whole IHNV virion

The G protein of IHNV was purified by column chromatography as described above. The column purified G protein, G_c, was lyophilized and reconstituted in 0.01 M Tris, pH 7.6, to 1 $\mu g/\mu l$. This protein was diluted in RPMI medium without serum and incubated with antiserum to IHNV in order to determine whether this protein would block the neutralizing activity of the anti-IHNV serum.

Results

Whole virus antiserum

Rabbit antiserum was prepared to purified IHNV and a comparison of its neutralizing activity was made to its reactivity to IHNV in ELISA, radioimmune precipitation and immunoblot assays (Table 1). For most rabbit antisera preparations the neutralization titers ranged from 1 : 200 to 1 : 250 (Hsu et al., 1985). In all cases the binding activity of the antisera was much greater than its neutralizing activity. In the ELISA assay, anti-IHNV sera could specifically detect IHNV antigen at a 1 : 32,000 dilution. The reaction was specific for IHNV since the anti-IHNV sera did not neutralize VSV or infectious pancreatic necrosis virus (IPNV) and did not react with these viruses by ELISA. In the immunoblot assay, IHNV proteins were detected by the anti-IHNV sera in the dilution range of 1 : 500 to 1 : 1000. All five viral proteins from [^{35}S]methionine labeled virus (Fig. 1) or in vitro translation products of IHNV mRNA (data not shown) were precipitated by this antiserum. The L band is not present in Fig. 1 because this lysate was obtained from cells early in infection (16 h post-infection) when low amounts of L protein were present (Leong et al., 1983).

Polyclonal monospecific antisera

Specific rabbit antisera were made respectively to G, N, M1 and M2 proteins of IHNV that had been purified and electroeluted from SDS-PAGE gels. The purity of each preparation was such that only a single band for each protein was detected by SDS-PAGE (Fig. 2). These proteins represented 95% of the total IHN virion protein. L, the presumed virion polymerase, represented 5% or less of the total

TABLE 1

Comparison of antisera to IHNV and IHNV proteins

Type of serum	Neutralization [a]	ELISA [b]	Immunoblot [c]
Anti-IHNV	1:200+	1:32000	1:750+
Anti-G_s [d]	1:40	1:16000	1:500
Anti-G_e [e]	1:40+	1:2000	1:400+
Anti-N	None	1:800	1:400
Anti-M1	None	1:8000	1:400
Anti-M2	None	1:16000	1:400+
Anti-N/Anti-M2	None	ND [f]	ND
Anti-N/Anti-G_e	1:40	ND	ND
Anti-M2/Anti-G_e	1:40	ND	ND
Anti-deG [g]	1:32	1:1600	1:400

[a] Neutralization dilution capable of 50-fold plaque reduction (98% neutralization).
[b] ELISA dilution capable of detecting 1 ng of virus.
[c] Immunoblot dilution capable of detecting 20 ng of protein per band.
[d] G_s, G protein was purified using differential solubility with 1%Triton X-100.
[e] G_e, G protein was purified by electroelution of the band from SDS-PAGE.
[f] ND, not done.
[g] deG, G protein that was deglycosylated enzymatically and electroeluted as above.

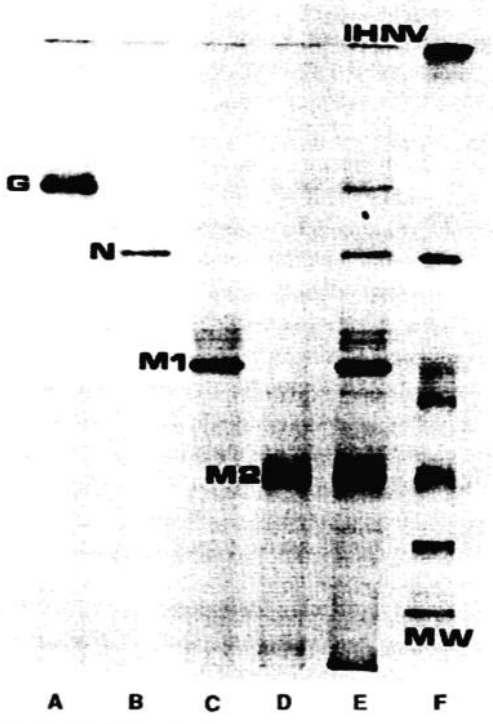

Fig. 1. Autoradiogram of a Radio-PAGE analysis of the immunoprecipitation of [^{35}S]methionine-labeled IHNV from infected cell lysates at 16 h post-infection with monospecific antisera. IHNV proteins labeled to left of lane. Radioimmunoprecipitation with: lane A, anti-IHNV G protein specific antiserum; lane B, anti-IHNV N protein specific antiserum; lane C, anti-IHNV M1 protein specific antiserum; lane D, anti-IHNV M2 protein specific antiserum; lane E, anti-IHNV polyclonal antiserum, L protein is not detectable early in infection; lane F, ^{14}C-labeled molecular weight markers (Amersham).

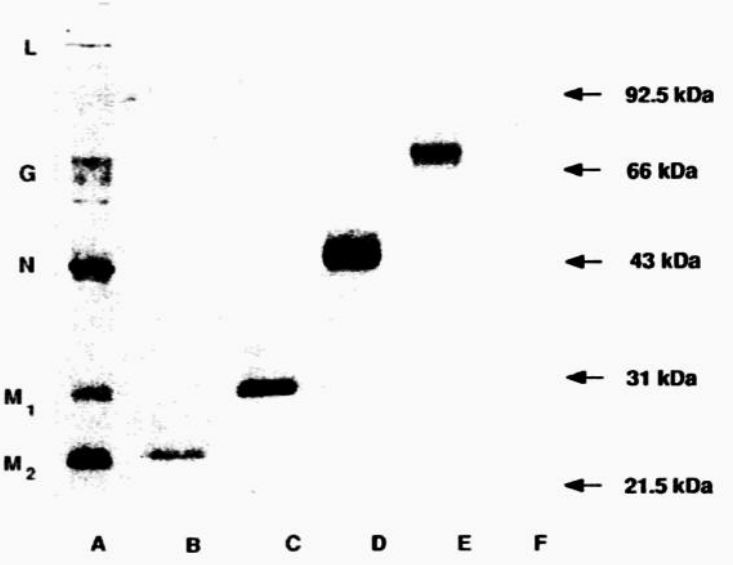

Fig. 2. SDS-10% PAGE of IHNV proteins purified by electroelution. Lane A, purified IHNV Round Butte isolate, RB1; lane B, electroeluted M2 protein; lane C, electroeluted M1 protein; lane D, electroeluted N protein; lane E, electroeluted G protein; lane F, electroeluted L protein, no visible band. The gel was stained with 0.1% Coomassie Brilliant Blue dye, destained and dried. IHNV proteins designated on the left side of lane A. All lanes contain 50 μl of sample except lane A, 5 μl and lane F, 100 μl. Molecular weights shown to the right side of lane F in descending order are phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor (BioRad).

structural proteins of IHNV (McAllister and Wagner, 1975; Hsu et al., 1986). Antiserum to the L protein was not made because an insufficient quantity of the protein was obtained by electroelution (Fig. 2).

The monospecific nature of the anti-G, -N, -M1 and -M2 antisera was determined by immunoblot and radioimmune precipitation assays. These monospecific antisera reacted only with their corresponding proteins and with titers that ranged from 1 : 200 to 1 : 750 (Table 1). The antigen for the immunoblot assays was purified IHNV. In radioimmune precipitation assays, anti-N, anti-M1, anti-M2, and anti-G antisera precipitated only the homologous IHNV protein of [^{35}S]methionine labeled virus (Fig. 1) or in vitro translation products of IHNV mRNA. All antisera reacted in ELISA procedures to the native proteins of IHNV (Table 1).

When used in serum neutralization assays, antiserum to N, M1 or M2 proteins were not neutralizing even at undiluted concentrations. When anti-N and anti-M2 antisera were mixed 1 : 1 (v/v) and tested at undiluted concentrations, no neutralization of IHNV was observed. Anti-G antiserum prepared against the electroeluted G (anti-Ge) did neutralize IHNV albeit at a titer that was approximately 2.5 times lower than that of anti-IHNV antiserum (Table 1). When anti-Ge antiserum was mixed 1 : 1 (v/v) with anti-N or anti-M2 antisera diluted 1 : 5, no enhancement of neutralization occurred.

Previous work with VSV and rabies virus had shown that antiserum directed against the virion glycoprotein was neutralizing; likewise, the anti-G antiserum prepared for IHNV was also neutralizing. However, anti-Ge antiserum had a neutralization titer 2–3 times lower than that of anti-IHNV antiserum. Thus, a monospecific antiserum to G protein which had been purified by differential solubility in Triton X-100 was prepared. G protein prepared in this manner was presumably less denatured than protein eluted from an SDS-PAGE gel and those immunogenic epitopes which might have been destroyed by boiling and reduction would be conserved. The anti-G antiserum against the detergent solubilized G protein (anti-Gs) did neutralize IHNV but its titer was as low as anti-Ge antiserum (Table 1). There was no significant difference between anti-Ge and anti-Gs antisera in neutralization or immunoblot assays. However, the anti-Gs antiserum reacted very well with native IHNV G protein by ELISA (Table 1) at 1 : 16,000.

G protein blocks neutralizing activity

The G protein of IHNV which had been solubilized by SDS treatment and purified by column chromatography was used in experiments to determine whether it would block the neutralizing activity of anti-IHNV antiserum. One or ten nanogram of column purified G protein, Gc, was mixed for 1 h at 15 °C with 1 : 50 dilutions of anti-IHNV antiserum or anti-G antiserum. In plaque reduction assays, the treatment of both antisera with Gc resulted in a complete loss of neutralizing activity. Complete blocking of neutralizing activity of the anti-IHNV antiserum, which was directed against all virion proteins, by the G protein provided strong evidence that only the anti-G component of anti-IHNV antiserum was neutralizing.

Glycosylation is not required to maintain neutralizing epitopes

Antiserum made to G protein that had been deglycosylated enzymatically with endoglycosidase F was tested for reactivity and neutralization capacity. Endoglycosidase F treatment removed all N-linked glycans as evidenced by the altered mobility of the treated IHNV G protein on SDS-PAGE (Fig. 3). No O-linked glycosylation occurred on the G protein (unpublished observation). The apparent molecular weight of the deglycosylated G protein was determined to be 55,300 by scanning laser densitometry analysis with a computer program, Videophoresis II (Biomed Instruments). This corresponded closely to the predicted molecular weight of the unglycosylated G protein of 56,795 daltons (Koener et al., 1987). Furthermore, the endoglycosidase F treated G protein comigrated with the G protein produced in infected cells treated with tunicamycin so that N-linked glycosylation was inhibited and with G protein produced by in vitro translation (unpublished observations). This antiserum was reactive to native purified IHNV in ELISA procedures and to denatured glycoproteins in immunoblot assays (Table 1). Furthermore, this antiserum also neutralized IHNV at about the same dilution as the other antisera directed to the G protein. This indicated that at least some, if not all, of the neutralizing epitopes did not require carbohydrates to maintain structural

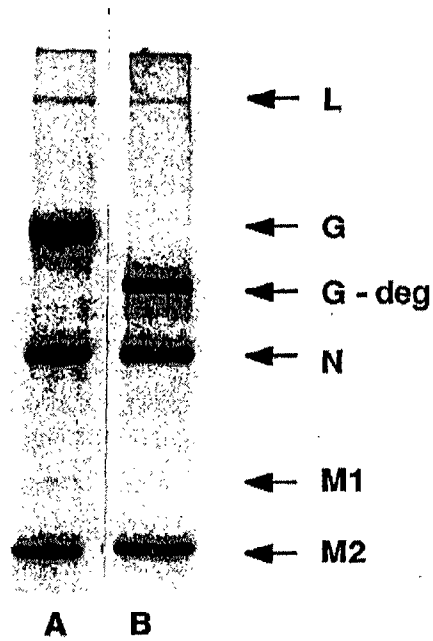

Fig. 3. Autoradiogram of a SDS-PAGE of endoglycosidase F treated [^{35}S]methionine-labeled purified IHNV. Lane A, purified IHNV. The five virion proteins, L, G, N, M, and M2 are indicated; lane B, endoglycosidase F treated IHNV. The deglycosidated G protein is shown as a faster migrating, G-deg, protein of about 55.3 kDa.

integrity and the neutralizing antibody response was directed towards the protein not the glycan portion of the G molecule.

Immunization of fish

Since only anti-whole virion and anti-G antisera showed in vitro virus neutralization, the protective effect of vaccinating fish with purified G protein was tested. The G protein was purified by Triton X-100 extraction and the detergent, which is toxic to fish, was removed by batch processing with SM-2 BioBeads. Groups of fish were vaccinated by immersion or injection. A series of six experiments were performed using rainbow trout and kokanee salmon fry obtained from different locations and at different times of the year. The procedures for immunization and virus challenge are outlined in Fig. 4. About thirty days post immunization fish were challenged with serial log dilutions of virulent IHNV. The challenge virus IHNV RB1 (Hsu et al.,1986) was isolated from steelhead trout dying of IHNV at Round Butte Hatchery and passed no more than three times in tissue culture. In all experiments (Tables 2 and 3) the immunized fish were protected from challenge by a lethal dose of IHNV. Kokanee were more susceptible to the Round Butte isolate of IHNV than rainbow trout. In experiments 1 to 4 (Table 2) the substantial protection afforded by G

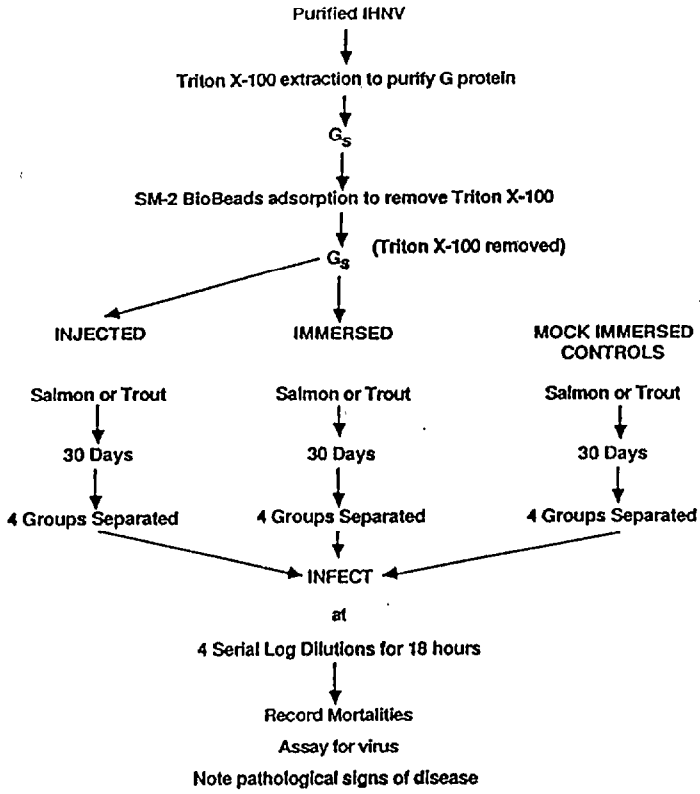

Fig. 4. Outline of salmonid fish immunization with G protein and challenge by IHNV.

protein immunization of kokanee salmon was demonstrated. At low concentrations of IHNV, the 10^{-4} and 10^{-5} challenge dilutions, similar to those found prior to an epizootic outbreak (Mulcahy et al., 1983), the immunization provided almost 100% protection.

Rainbow trout exhibited a similar pattern of protection when immunized by the G protein (Table 3). However, lower overall mortality occurred in the rainbow trout. At the lower concentrations of IHNV, the relative percent survival, RPS, value (Johnson, 1982a, b), was not meaningful because there were too few deaths in all groups (Table 3).

The LD_{50} (lethal dose to 50% of the test fish) for kokanee salmon ranged from 1.5×10^2 to 5.3×10^3 $TCID_{50}$/ml and for rainbow trout from 9.1×10^2 to 5.1×10^3 $TCID_{50}$/ml (Tables 2 and 3). The LD_{50} for kokanee salmon immunized by either method ranged from 2.8×10^3 to 2×10^5 $TCID_{50}$/ml (Table 2) and for immunized rainbow trout from 6.8×10^4 to 2.2×10^6 $TCID_{50}$/ml (Table 3). The average amount of virus to achieve an LD_{50} for kokanee salmon and rainbow trout that were immunized by immersion was 54 times that of control fish. When the fish were

TABLE 2

Protection of kokanee salmon fry from virulent IHNV by G protein immunization

	Log virus/ dilution [a]	Inoculated				Immersed				Control		
		N	SL	%	RPS	N	SL	%	RPS	N	SL	%
Exp. 1 [b]	−2	25	8	32	62	25	17	68	19	25	21	84
	−3	25	3	12	80	25	6	24	60	25	15	60
	−4	25	0	0	100	25	1	4	92	25	12	48
	−5	25	0	0	100	25	0	0	100	25	8	32
LD_{50}			$>1\times10^{5}$ [c]				3.9×10^{3}				1.5×10^{2}	
Exp. 2 [b]	−2	25	14	56	44	25	18	72	28	25	25	100
	−3	25	5	20	80	25	7	24	72	25	25	100
	−4	25	0	0	100	25	2	8	86	25	14	56
	−5	25	0	0	100	25	0	0	100	25	3	12
LD_{50}			4.9×10^{4}				2.5×10^{4}				5.3×10^{3}	
Exp. 3 [b]	−3	24	18	75	25	25	23	92	8	32	32	100
	−4	26	13	50	50	25	15	60	40	37	37	100
	−5	22	1	4.5	93	25	5	20	70	30	20	67
	−6	23	0	0	100	27	3	11	35	30	5	17
LD_{50}			5×10^{4}				2.8×10^{3}				2.3×10^{2}	
Exp. 4 [b]	−2	25	2	8	89	25	1	4	94	25	18	72
	−3	25	0	0	100	25	4	16	71	25	14	56
	−4	25	0	0	100	25	0	0	100	25	3	12
	−5	25	3	12	ND	25	0	0	ND	25	0	0
LD_{50}			$>2\times10^{5}$ [c]				$>2\times10^{5}$ [c]				1.5×10^{3}	

[a] Serial log dilutions of infectious low passage IHNV for challenge.

[b] Challenge IHN virus titers for experiments as follows: 1, 1×10^{7} $TCID_{50}$/ml; 2, 7.2×10^{8} $TCID_{50}$/ml; 3, 5×10^{8} TCID/ml; 4, 2×10^{7} $TCID_{50}$/ml.

[c] Estimated since an LD_{50} did not occur in these groups.

Abbreviations used: SL, specific loss, i.e. number of fish dying from IHNV infection; %, percent mortality; RPS, relative percent survival (Johnson et al., 1982a); LD_{50}, lethal dose of IHNV in $TCID_{50}$/ml to cause 50% mortality; ND, not determinable due to no mortalities in control group.

TABLE 3

Protection of rainbow trout from virulent IHNV by protein immunization

	Log virus/ dilution [a]	Inoculated				Immersed				Control		
		N	SL	%	RPS	N	SL	%	RPS	N	SL	%
Exp. 5 [b]	−2	25	4	16	78	25	4	16	78	25	18	72
	−3	25	3	12	77	25	4	16	69	25	13	52
	−4	25	1	4	0	25	0	0	100	25	1	4
	−5	25	0	0	ND	25	0	0	100	25	0	0
LD_{50}			$>1\times10^{5}$ [c]				$>1\times10^{5}$				9.1×10^{2}	
Exp. 6 [b]	−2	25	12	48	33	25	15	60	17	25	18	72
	−3	25	10	40	60	25	10	40	60	22	22	100
	−4	24	5	20	0	25	4	16	20	25	5	20
	−5	25	1	4	0	21	3	12	0	25	1	4
LD_{50}			2.2×10^{6}				6.8×10^{4}				5.1×10^{3}	

[a] Serial log dilutions of infectious low passage IHNV for challenge.

[b] Challenge IHN virus titers for experiments as follows: 5, 1×10^{7} $TCID_{50}$/ml; 6, 2.2×10^{8} $TCID_{50}$/ml.

[c] Estimated since an LD_{50} did not occur in these groups.

For explanation of the abbreviations used, see legend to Table 2.

injected with G protein, about 166 times the amount of IHNV was required to achieve an LD_{50} as compared to unimmunized controls.

Discussion

Four of the five structural proteins, N, M1, M2 and G, of IHNV were purified and used to prepare monospecific antisera in rabbits (Fig. 2, Table 1). Only the envelope glycoprotein, G, elicited a neutralizing antibody response in rabbits (Table 1). Antisera prepared to N, M1 and M2 proteins had no neutralizing or neutralizing enhancement activity. Furthermore, the neutralizing activity of antiserum prepared to the whole virus, which reacts with all five virion proteins in immunoblot and other assays (Table 1 and unpublished observations), was blocked by incubation with purified G protein. This result discounted the possibility of any neutralizing response to the L protein to which monospecific antiserum could not be made. It appeared that the G protein of IHNV as with the G protein of the mammalian rhabdoviruses, VSV and rabies virus, was solely responsible for inducing neutralizing antibody.

Interestingly, although the G protein was subjected to denaturation with heat, detergent and reducing agents, epitopes were preserved that elicited a neutralizing antibody response. This antiserum also recognized the native but unglycosylated form of the G protein as shown by the immune precipitation of in vitro translated IHNV mRNA products. The function(s) of the carbohydrate moieties on the G protein of IHNV is unknown; however, this result indicated that the formation of at least some antigenic determinants did not require glycosylation. Glycosylation is necessary for proper tertiary structure formation of some proteins (Kornfeld and Kornfeld, 1985). In VSV, glycosylation may function to 'hide' epitopes from antibodies (Vandepol et al., 1986). Likewise, variants of rabies virus, which escape neutralization by anti-rabies monoclonal antibodies, have additional glycosylation sites which may act to cover (mask) epitopes and render them inaccessible to these antibodies (Wunner et al., 1985).

The capacity of the G protein to induce protective immunity in the natural host, salmonid fish, was tested in kokanee salmon and rainbow trout fry. Protective immunity was elicited by both immersion or injection methods of vaccination. Thirty days post immunization the fish were protected from waterborne challenge by virulent IHNV. The LD_{50} of IHNV for unimmunized kokanee salmon varied from 1.5×10^2 to 5.3×10^3 $TCID_{50}$/ml and for control rainbow trout from 9.1×10^2 to 5.1×10^3 $TCID_{50}$/ml (Tables 2 and 3). This finding was within the range that had been reported previously (Amend and Nelson, 1977; Chen, 1984). The kokanee salmon immunized by immersion required 4 to 140 times as much IHNV to reach an LD_{50} as the controls. The immersed vaccinated rainbow trout required 13 to 100 times as much IHNV to kill 50% of the fish. The inoculated vaccinated kokanee salmon required 15 to 300 times as much IHNV as the controls to reach an LD_{50}. Injection was approximately two-fold more protective than immersion in kokanee salmon. The rainbow trout that were injected with G protein

required 110 to 420 times as much IHNV to produce an LD_{50} (Tables 2 and 3). For rainbow trout, injection conferred about three times greater protection than immersion. There was no significant difference in vaccination treatments at environmental levels of IHNV, which were reproduced at the 10^{-4} and 10^{-5} challenge dilutions (Mulcahy et al., 1983). Also, at these lower levels of IHNV, almost complete protection of fish was conferred by G protein immunization (Tables 2 and 3). The RPS values in almost all cases were greater than 60% (Tables 2 and 3), which was considered the minimum level of protection necessary for vaccination to be effective in disease outbreaks (Johnson et al., 1982b). Thus, significant protection to fish in production facilities from IHNV could be attained by this type of immunization.

Comparison with protein dosage used in the rabies studies suggests that the IHNV glycoprotein is less immunogenic in fish. In rabies studies, dosages of 12.5 μg of purified G protein per adult mouse (30–35 g body weight) or 50 μg of G protein per rabbit (2000 g body weight) were used to give complete protection (Cox et al., 1977). The salmonid fish in these studies were injected with 0.4 to 0.5 μg of IHNV G protein or 100 fish were immersed in 4 ml of 40–60 μg/ml of G protein solution. The protein dose: body weight ratio used is about 0.5 μg/0.5 g fish or a protein dosage ratio of $1/10^3$ for the inoculated fish.

With rabies virus the protein dosage ratio to give complete protection ranges from $1/2 \times 10^6$ to $1/40 \times 10^6$ protein immunogen to body weight. The 10^3 to 10^4 difference in the protein dose may be accounted for by several factors. First, these salmonid fish were very young, 1–3 months old, and small, less than 1 g in body weight, and were not fully competent immunologically (Johnson et al., 1982a). Johnson and colleagues (1982a, b) found that salmonids less than 1 g in size responded poorly to bacterins and their immunity was of limited duration. However, IHN disease is a disease of young fish and must be tested on such small susceptible fish (Pilcher and Fryer, 1980). The second reason for the poor immunogenicity of the G protein might be disaggregation of G protein homopolymers. The VSV glycoprotein spike is thought to be a multimer of G proteins (Dubovi and Wagner, 1977). This structure is presumed to be necessary for preservation of antigenic sites and induction of immunity in vivo. Non-denatured purified G protein of rabies virus forms rosette structure as determined by electron microscopy and is highly immunogenic. Monomeric forms of G protein of rabies virus produced in Triton X-100 are at least ten times less protective than inactivated virus vaccines (Wunner et al., 1983). When NP-40 detergent extraction is used, the G protein is 9 to 24 times less protective (Cox et al., 1977).

The soluble form of the rabies virus glycoprotein Gs, which misses 58 C-terminal amino acids, does not aggregate and is weakly protective as a vaccine. It is at least 30 times less protective (Dietzschold et al., 1983). Finally, analysis of CNBr fragments of rabies virus G protein and the major neutralization epitope of VSV (Indiana) designated the epitope A suggests that these epitopes are formed by both contiguous and noncontiguous regions of the glycoprotein (Wunner et al., 1983; Vandepol et al., 1986). This finding is consistent with a fold back model of the protein where G protein forms an intramolecular coiled coil (Crimmins et al., 1983).

When the tertiary structure of the A epitope of VSV was disrupted, the denatured protein was 100 times less immunogenic. A computer-generated secondary structure model of the IHNV G protein suggested foldbacks and coiling might occur in the tertiary structure of this protein also (Koener et al., 1987). In the presence of detergent, the IHNV G protein might lose some of its normal configuration. Any of these reasons might account for the lower immunogenicity of the purified IHNV G protein. The minimum required immunization dose has not been determined. Protection, however, was achieved by using the G protein preparation and indicated the suitability of immunizing small salmonid fish to IHN disease. IHN disease has been shown to require relatively high titers of virus (about 10^5 PFU/g of gill tissue, $1-5 \times 10^5$ $TCID_{50}$/ml) in the gills to cause systemic infections (Mulcahy et al., 1983). In these experiments the LD_{50} was achieved at roughly $1-2 \times 10^5$ $TCID_{50}$/g of fish (Tables 2 and 3). Thus, protection was afforded at virus concentrations similar to that found in hatcheries where there are outbreaks of the disease. The immunity conferred by the G protein vaccination may increase the effectiveness of the gill barrier to IHNV and be useful in preventing natural IHNV outbreaks.

The results presented here suggest that a subunit vaccine based on the IHNV G protein is feasible. Other investigators have developed a hybridoma that produced a neutralizing G-specific immunoglobulin that confirmed these results (Arakawa et al., 1986; Winton et al., 1988). A portion of the G protein gene has been expressed in an *E. coli* system and this protein was immunogenic and protective (Gilmore et al., 1988). Experiments to determine the cross-protection and neutralizing capabilities of the G protein from this Type 1 IHNV isolate to challenge by other biochemically distinct isolates of IHNV are in progress.

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MANUSCRIPT # 10

Expression of the Glycoprotein Gene from a Fish Rhabdovirus by Using Baculovirus Vectors

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Expression of the Glycoprotein Gene from a Fish Rhabdovirus by Using Baculovirus Vectors†

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A cDNA fragment containing the gene encoding the glycoprotein of infectious hematopoietic necrosis virus was inserted into *Autographa californica* baculovirus vectors under the control of the polyhedrin promoter. A 66-kilodalton protein, identical in size to the glycosylated glycoprotein of infectious hematopoietic necrosis virus, was expressed at high levels in *Spodoptera frugiperda* cells infected with the recombinant viruses. The expressed protein reacted with antiserum to the glycoprotein on Western blots (immunoblots).

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus which infects young salmon and trout (9). Over the last 30 years, IHNV has caused extensive mortality in hatchery fish populations in the Pacific Northwest of the United States. Currently, control methods for the disease are limited to avoidance procedures which consist of the destruction of the infected fish and quarantine of all other fish in the hatchery.

The development of a vaccine against IHNV would be of great practical and economical value. The safety of live attenuated vaccines has been questioned in the case of IHNV because virus transmission occurs via the water (8). Since the virion surface glycoprotein of IHNV is immunoprotective (1), a subunit vaccine consisting of the glycoprotein was considered as an alternative.

In a previous report, we described the cloning and sequencing of a cDNA of the IHNV glycoprotein. The cDNA of the IHNV glycoprotein gene is 1,609 base pairs long and encodes a protein of 508 amino acids, which translates into a molecular weight of 56,795 (3).

Recombinant baculoviruses are capable of expressing proteins at high levels (6). In addition, because expression occurs in insect cells, the proteins appear to be processed in a eucaryotic manner. To determine if the baculovirus system is capable of expressing the IHNV glycoprotein, we have constructed recombinant baculoviruses carrying the IHNV glycoprotein gene.

The ability of baculovirus expression systems to produce IHNV glycoprotein was assessed with four recombinant baculoviruses. The IHNV glycoprotein gene was ligated into the transfer vectors pAc611 and pAc373 that were provided by M. D. Summers, Department of Entomology, Texas A & M University, College Station, Tex. The following constructions were used (Fig. 1): pAc611-G1, pAc611-G2, pAc373-G3, and pAc373-G4. In constructing pAc611-G1, the glycoprotein gene was excised from the pT7-2 plasmid with *Pst*I and ligated into the *Pst*I site of the vector pAc611. This construct contained the entire glycoprotein cDNA, including 12 G residues from the cDNA cloning protocol and an untranslated leader sequence of 48 nucleotides at the 5′ end. The clones pAc611-G2, pAc373-G3, and pAc373-G4 were constructed to eliminate these untranslated sequences, because it was thought that they might inhibit efficient expression of the glycoprotein. In constructing pAc611-G2, the glycoprotein gene was removed from its parent plasmid with *Bst*XI and *Hin*dIII and cloned into the *Sma*I site of pAc611. The *Bst*XI site was located at the first ATG of the glycoprotein coding sequence. In constructing pAc373-G3, the glycoprotein gene was first subcloned into the plasmid vector pUC19 and, in a second cloning step, it was put into the vector pAc373. In constructing pAc373-G4, the glycoprotein gene was cut with *Bst*XI and *Hin*dIII and blunt ends were created to clone it into the unique *Bam*HI site of pAc373.

In all four plasmid constructions, the 3′ end was cut from inside the parent plasmid pT7-2 with either *Pst*I or *Hin*dIII, leaving a noncoding region of 34 nucleotides after the TAA stop codon at position 1573. This stop codon should be utilized in the baculovirus system. However, changes in the 5′ end of the glycoprotein gene might affect the expression process and expression levels (6). The 5′ end of the glycoprotein gene was different in each of the four plasmid constructions. The recombinant plasmid, pAc611-G1, has 12 G residues at the 5′ end followed by a noncoding region of 48 nucleotides, and the two ATG sites at positions 49 and 58 are the same as in the original cDNA clone (ACAATGGACAC CATGA) (3).

In the three other plasmid constructions, the 5′ end occurred at the *Bst*XI site of the glycoprotein gene so that the noncoding nucleotide residues were removed and the glycoprotein gene sequence started at the first ATG. When ligated into the *Sma*I site of pAc611 or pUC19, the 5′ end sequence would read CCCATGGACACCATGA. This is the case for pAc611-G2 and pAc373-G3. The fourth plasmid construction, pAc373-G4, has the glycoprotein gene cloned into the blunt end of the *Bam*HI site and its 5′ start sequence was GGATCATGGACACCATGA. Both ATG codons at the beginning of the glycoprotein gene reside at the same reading frame and the initial ATG is the presumptive start codon. When the first ATG start sites for each plasmid construction were analyzed for the consensus translational initiation sequence, ACCATGG (4), all had the requisite G in the +4 position. pAc611-G1 and pAc373-G4 have a purine (A) in the −3 position which is favored over the C in the −3 position in the two other plasmid constructions. This analysis indicated that expression of the IHNV glycoprotein in the baculovirus system with these plasmids was theoretically possible.

The IHNV glycoprotein gene in the recombinant plasmid was then transferred into the wild-type *Autographica cali-*

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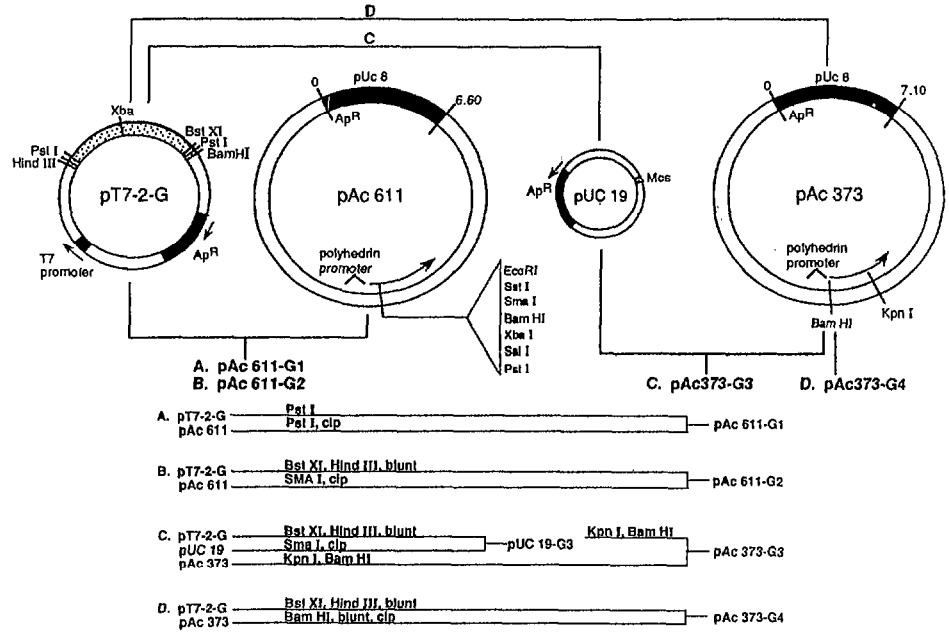

FIG. 1. Construction of recombinant baculovirus vectors containing the glycoprotein gene of infectious hematopoietic necrosis virus. pAc611-G1 (A) contains the glycoprotein gene in the *Pst*I site of pAc611. pAc611-G2 (B) contains the glycoprotein gene in the *Sma*I site of pAc611. pAc373-G3 (C) contains the glycoprotein gene between the *Bam*HI and *Kpn*I sites of pAc373. pAc373-G4 (D) contains the glycoprotein gene in the *Bam*HI site of pAc373. The restriction maps of pAc611 and pAc373 were modified from versions of those described by Summers and Smith (10). ▨, Glycoprotein gene; ■, ampicillin resistance gene and T_7 promoter.

fornica nuclear polyhedrosis virus (Ac*M*NPV) genome by homologous recombination within insect cells. To accomplish this, *S. frugiperda* Sf9 cells were cotransfected with 2 μg of Ac*M*NPV DNA and 3 μg each of the recombinant baculovirus vectors (10). Plaques produced from each transfection were screened under the microscope for polyhedrin-negative phenotypes. Plaque hybridizations with a glycoprotein gene-specific probe labeled with ^{32}P by nick translation were performed to confirm the results. After a second cycle of plaque purification and plaque hybridization, recombinant viruses containing the glycoprotein gene were isolated from the infected insect cells and stocks of the recombinant viruses were obtained through infection of insect cells.

Monolayers of *S. frugiperda* cells were infected at high multiplicity with Ac*M*NPV or the recombinant baculoviruses. At 3 and 6 days postinfection, the infected cells were lysed and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5) (Fig. 2). All four recombinants synthesized a major protein species that migrated with an estimated size of 66,000 daltons, similar to that of the IHNV glycoprotein (7). A densitometer scan of the gel shown in Fig. 2 indicated that the quantity of glycoprotein produced by infected cells was 20 to 30% of the total protein (data not shown).

The virus-produced protein was analyzed by immunoblotting with rabbit anti-IHNV glycoprotein serum (Fig. 3). Lanes containing expressed glycoprotein all reacted positively with the antiserum, whereas lanes containing mock-infected or Ac*M*NPV-infected samples did not show a reaction with the antiserum. The size of the protein as determined by gel electrophoresis corresponded to the size of the glycosylated form of the glycoprotein (molecular weight, 66,000), as opposed to the molecular weight of the nonglycosylated form, whose predicted molecular weight was 56,795. There are five potential *N* glycosylation sites in the glycoprotein DNA sequence (3). The vector difference as well as the differences in the 5′ ends of the constructs appeared to have little or no effect on the level of expression of the glycoprotein in this system.

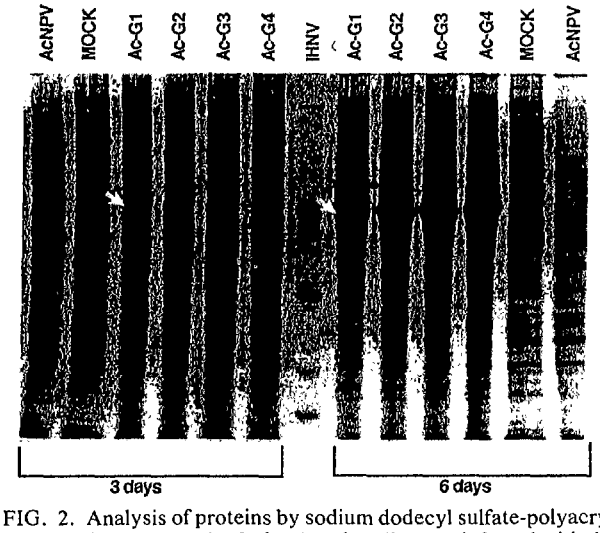

FIG. 2. Analysis of proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *S. frugiperda* cells were infected with the following recombinant baculoviruses: Ac611-G1, Ac611-G2, Ac373-G3 and Ac373-G4. Cell lysates were analyzed by electrophoresis on a 9% polyacrylamide gel (5) and stained with Coomassie brilliant blue. Samples of cells infected with wild-type Ac*M*NPV and mock-infected cells were included for comparison. Lane 7 (designated IHNV) contains purified IHNV proteins whose molecular weights are in the following order of size: L, 150,000; G, 66,000; N, 40,000; M1, 25,000; and M2, 21,000. The arrows indicate the positions of the expressed IHNV glycoprotein.

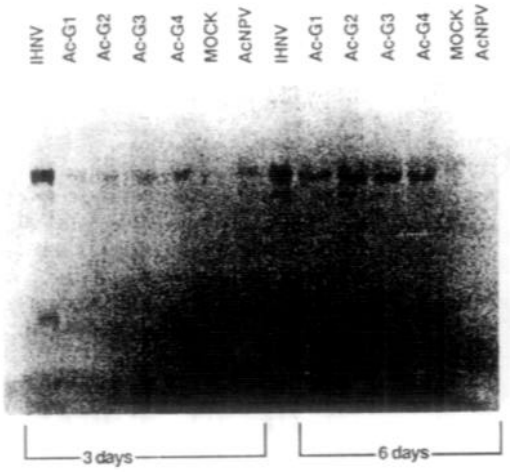

FIG. 3. Analysis of proteins by Western immunoblot. *S. frugiperda* cells were infected with recombinant or wild-type Ac*M*NPV or were mock infected as described in the legend to Fig. 2. In this gel, the samples for the 6-day cell lysates contained 1/10 of that represented in Fig. 2. Purified IHNV proteins were included as a positive control. After electrophoresis, the proteins were blotted onto a nitrocellulose membrane. The blot was incubated with a 1:250 dilution of rabbit anti-IHNV glycoprotein serum. The bound antibody was detected by using goat anti-rabbit immunoglobulin G horseradish peroxidase complex (Sigma Chemical Co., St. Louis, Mo.) with 4-chloro-1-naphthol as substrate.

Immunoprecipitation experiments showed that the glycoprotein was not present in detectable amounts in the tissue culture supernatant (data not shown). Only cell lysates contained the expressed protein. This finding suggested that the glycosylated glycoprotein was not secreted by the insect cells. It was more likely that the expressed protein remained on the infected cell surface, as has been reported for a number of other foreign proteins (6). The plasmid constructions used in this study retained the signal peptide, transmembrane domain, and cytoplasmic tail of the glycoprotein. Preliminary data on fluorescent antibody staining of the infected cells with anti-IHNV sera show surface expression of the glycoprotein.

Among the proteins expressed in the baculovirus system that undergo subsequent *N*-glycosylation were many viral proteins such as the glycoprotein of human immunodeficiency virus, the hemagglutinin of influenza virus, the neuraminidase of parainfluenza virus, and the glycoprotein precursor of lymphocytic choriomeningitis virus (for a review, see reference 6). All of these glycoproteins were expressed in the baculovirus system because it provided appropriate posttranslational modifications and expressed levels of protein of up to 500 μg/ml per 10^6 cells at 72 h postinfection. Similarly, the IHNV glycoprotein was expressed in the Ac*M*NPV vector-insect cell system to provide large quantities of the protein for vaccine development.

We have identified a region of the IHNV glycoprotein that induces protective immunity in young salmon and trout (2). Although this vaccine (a crude extract of *Escherichia coli* harboring a plasmid expressing a trpE-glycoprotein segment fusion protein) was effective with many different strains of the virus, only a small segment of 350 bases from the glycoprotein gene was expressed. The 16 cysteine residues present in the glycoprotein made expression of the entire protein in native form in *E. coli* unlikely. Certain virus strains might not contain epitopes cross-reactive with this region of the gene. Thus, the synthesis of the entire IHNV glycoprotein in glycosylated form in insect cells makes possible the development of a more complete IHNV vaccine. The efficacy of the recombinant baculovirus-produced glycoprotein as a vaccine has yet to be tested in vivo.

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MANUSCRIPT # 11

Potential Uses of Recombinant DNA in the Development of Fish Vaccines

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POTENTIAL USES OF RECOMBINANT DNA IN THE DEVELOPMENT OF FISH VACCINES

By J.-A. Leong* and C.B. Munn**

The Need for New Approaches to Vaccination

Vaccination ranks as one of the greatest successes of medicine, with some of the planet's most pernicious diseases eradicated or well controlled. Yet, despite this success, technical limitations have so far restricted the number of commercially-produced human vaccines to twenty and veterinary vaccines to about fifty. Many microbial pathogens continue to present serious problems, but recent advances in biotechnology signal the real promise of increasing substantially our success in producing new vaccines.

There are three principal approaches to vaccination. The simplest is the use of whole killed bacteria or inactivated viruses. This has led to many successful human and veterinary vaccines. The major problems with this approach are (a) the protective antigens are often destroyed during inactivation (b) unwanted side-effects and antigenic competetion may occur as the result of the complex mixture of microbial products present and (c) the correct type of immune response (i.e. humoral or local antibody, or cell-mediated) may not always be induced. Thus, the vaccines produced by this approach are often far from perfect, and there is considerable interest in finding new alternatives. In the case of fish diseases, this simple approach has led to the commercial development and wide use of some very successful bacterial vaccines (notably against vibriosis and enteric redmouth), but in most other cases, including virus diseases, killed vaccines have proved of doubtful efficacy or have been uneconomic and impractical to administer.

A second method is to use a live vaccine in which virulence has been attenuated by repeated *in vitro* culture or by the use of mutagens. This has been highly successful, the principal advantage being that the important protective antigens are delivered, via *in vivo* microbial replication, to the appropriate site and in the form needed to stimulate the correct immune response. Live vaccines are particularly suitable for oral administration, which has many advantages. Although superior, the major problem with live attenuated vaccines is the possibility of reversion to a virulent form. Many attenuated virus vaccines (e.g. Sabin polio and Rubella) have proved to be stable and safe for human use, but fear of reversion by mutation or recombination with the natural gene transfer has prevented the acceptance of all but a few bacterial vaccines of this type (e.g. BCG tuberculosis vaccine). Modern techniques provide new opportunites for the development of attenuated vaccines. For example, it is possible to use cloning techniques to ensure that genetic information encoding a key virulence factor is completely and permanently deleted. An example of the use of this approach is in the development of a live cholera vaccine, in which the genes encoding production of the A-subunits of the enterotoxin have been manipulated and replaced by genes encoding antibiotic or mercury resistance (Kaper *et al.*, 1984). Other approaches have been used in the development of new live vaccines against typhoid. Strains of *Salmonella typhi* which are deficient in galactose epimerase have been produced and shown to be safe and effective in human trials (Levine *et al.*, 1987). In these strains, the particular metabolic defect means that the bacteria are capable of very limited multiplication in the gut, resulting in delivery of the protective antigens, before the bacteria 'self-destruct'. A more generally applicable method may

be the use of genetically-engineered bacterial mutants which are defective in parts of the pathway for aromatic amino acid metabolism (Hoiseth & Stocker, 1981). Because this pathway is unique to bacteria, there is no possibility of reversion or supplementation *in vivo*, and vaccines based on such mutants of *Salmonella typhimurium* are being intensively researched (Curtiss *et al.*, 1988; Dougan, 1989). It is important to stress that recombinant DNA technology is not essential to these developments - for example, the Ty21A *S.typhi* live vaccine strain (Germanier & Furer, 1975) and prototype live vaccines against rotaviruses (Channock *et al.*, 1975) have been produced using 'traditional' technology. However, there can be little argument that the new techniques increase the available options considerably, and there is no exception in the case of fish vaccines. However, the concerns about the use of live vaccines in aquaculture and, in particular, the release of genetically-engineered organisms into the aquatic environment pose a number of difficulties which will be considered below.

The third approach to vaccine production is the use of subunits. Here, microbial antigens which have an important role in the pathogenic process (usually surface proteins or toxins) are purified from cultured bacteria or viruses. In the case of bacterial toxins, it is necessary to render the product non-toxic and some toxoid vaccines have been highly successful in the control of tetanus and diphtheria; such vaccines being developed in the complete absence of sophisticated molecular techniques. However, such straightforward examples are rare, and it is only through an understanding of the molecular basis of pathogenic processes that progress in many other diseases will be possible. For example, recombinant vaccines directed against the toxins of *Bordetella pertussis* (Arciniega *et al.*, 1987) and *Vibrio cholerae* (Holmgren & Svennerholm, 1985) have recently become possibilities. A notable example among the virus diseases is hepatitis B. The traditional vaccine has been produced by isolating the 22 nanometre protein virus subunit from the plasma of chronically-infected humans, followed by extensive purification. This expensive process has now been replaced by a recombinant vaccine in which the appropriate viral genes have been cloned in a variety of expression systems including bacteria, yeast, insect and mammalian cells (Hilleman, 1988). Once cloned, it becomes possible to use various vectors to deliver the subunit antigens. For example, modified vaccinia virus (Moss *et al.*, 1988) and aro- *S.typhimurium* (Dougan, 1989) are both being developed to carry a 'cocktail' of antigens for human use. A further advance has become possible with the use of advanced techniques for sequencing genes and synthesising synthetic oligonucleotides, and by the use of monoclonal antibodies for the delineation of epitopes in the cloned protein. Thus, the next generation of vaccines may be synthetic peptides which are immunogenic. Modification of sequences leads to the concept of protein engineering, and the so-called 'designer vaccines'. It is obvious that cloning of subunits offers many advantages in the development of new fish vaccines, especially where the agent is difficult to culture, where microbial products are produced naturally in small amounts or where purification (for example, to remove toxins) is uneconomic. In the case of multicellular parasites, recombinant vaccines may offer the only prospect of progress. Successful implementation of this approach depends on identification of the protective antigens and successful delivery of the pure antigens in a form to which the fish will mount an effective immune response (Ellis, 1988).

Against this background, we will now examine examples of the successful use of recombinant DNA technology and future prospects in the development of

vaccines for control of fish diseases.

Development of a Recombinant Vaccine for IHN

Viral diseases in aquacultured species have been particularly troubling for the industry. When an outbreak occurs, the only response available for many farmers has been the destruction of the fish in the entire facility. Thus, a safe, inexpensive antiviral agent is needed by the aquaculture industry.

Infectious hematopoietic necrosis virus (IHNV) is a very serious pathogen of rainbow trout, steelhead trout, and chinook salmon in the Pacific Northwest region of North America. In fact, it has been identified as the number one pathogen in the Columbia River Basin. Efforts have been made to develop a vaccine against IHNV with a killed preparation, an attenuated vaccine, and more recently, a subunit vaccine generated by recombinant DNA technology (Leong *et al.*, 1988). The killed vaccine has not been considered seriously by researchers because it is relatively expensive to prepare and appears to be effective only if administered by inoculation. The attenuated vaccines that have been developed were shown to be very effective in some fish species and lethal in other species. Furthermore, fish pathologists have questioned whether a live IHNV vaccine might introduce a measure of uncertainty in determining whether fish populations have had previous contact with the virus. The most compelling argument against the use of live attenuated vaccines for IHNV is based upon the fact that IHNV is an RNA virus with a high mutation rate and the possibility that such an attenuated virus may revert to virulence is a real one.

A prototype subunit vaccine for IHNV has been developed from the cloned gene for the IHN viral glycoprotein (Kurath *et al.*, 1985; Koener *et al.*, 1987). In this case, a segment of the viral glycoprotein gene was ligated in frame to the *trpE* gene of *Escherichia coli* and this fused *trpE*/IHNV glycoprotein construction was transformed into the bacteria. The fused DNA was placed in a plasmid that grew in bacteria and permitted the expression of the viral glycoprotein in the new host. When the bacterially produced viral protein was used to immunize fish, very good protection was observed (Gilmore *et al.*, 1988). A crude bacterial lysate was all that was required for vaccination by immersion and fish as small as 0.4 g were capable of responding to the vaccine.

Although there is only one serotype identified for IHNV by serum neutralization with polyclonal rabbit anti-IHNV sera (McCain *et al.*, 1971; Engelking, 1988), concerns have been raised regarding vaccine efficacy with all strains of the virus. The subunit vaccine has been shown to induce protection against at least six different isolates of IHNV. These isolates include virus taken from a number of different salmonid species (chinook salmon, kokanee salmon, and rainbow trout) as well as from a wide range of geographic locations. It appears to confer protection in all cases in laboratory trials.

Prospects for other Viral Fish Vaccines

The viral diseases that might be considered serious pathogens in the aquaculture industry are described in Table 1. The list includes several rhabdoviruses, herpesviruses, a birnavirus and a baculovirus. All of these agents have an economic impact on their respective cultured hosts and are likely candidates for vaccine development.

The rhabdovirus, viral hemorrhagic septicemia virus (VHSV), kills rainbow trout at the fingerling to yearling stage of life and is considered an economically important pathogen because it does kill larger, older fish. The virus is very similar to IHNV in that it is a probable

Table 1 Viral Pathogens of Economic Importance in Aquaculture

VIRUS	FAMILY	HOST	VACCINE UNDER DEVELOPMENT
Infectious hematopoietic necrosis virus (IHNV)	Rhabdoviridae	Salmon Trout	Subunit, live attenuated
Viral hemorrhagic septicemia virus (VHSV)	Rhabdoviridae	Salmon Trout	Subunit, live attenuated
Spring viremia of carp virus, pike fry rhabdovirus, grass carp rhabdovirus	Rhabdoviridae	Carp Pike	Killed, live attenuated
Infectious pancreatic necrosis virus (IPNV)	Birnaviridae	Salmon Trout Eel	Killed, live attenuated, subunit
Herpesvirus ictaluri	Herpesviridae	Catfish	Live attenuated
Fish pox herpesvirus	Herpesviridae	Carp	None
Baculovirus monodon	Baculoviridae	Shrimp	None
Infectious hypodermal and hematopoietic necrosis virus		Shrimp	None

member of the lyssavirus genus of Rhabdoviridae and it also has only one serotype by neutralization assays. An attenuated vaccine for VHS has been developed by P. de Kinkelin and his colleagues in France. However, the attenuated vaccine has not been licensed because of potential residual virulence and the concerns of state authorities that a live attenuated vaccine might revert to virulence for other fish species in the watershed (de Kinkelin, 1988). A subunit vaccine for VHS is under development by investigators at Eurogentec, Inc. in Belgium (Thiry *et al.*, 1989) and at the National Veterinary Laboratory in Denmark (P. Vestergard Jorgensen, personal communication). The VHS glycoprotein gene has been cloned and the entire gene has been expressed in yeast. This VHS subunit vaccine is being tested in fish now.

The rhabdoviruses, pike fry rhabdovirus (PFRV) and grass carp rhabdovirus (GRV), are serologically indistinguishable (Clerx & Horzineck). More recently, these two viruses were shown to be related to another rhabdovirus, spring viraemia of carp virus (SVCV), and it appears that SVCV and PFRV are representatives of two serotypes of one virus species (Vestergard-Jorgensen *et al.*, 1989). These rhabdoviruses induce systemic infections with generalized viremia and hemorrhages in viscera and muscles in pike, grass carp, and common carp. SVCV is endemic in European countries which raise carp and outbreaks of the viral disease vary from the usual 30-50% to as high as 70%. A commercial vaccine produced by Bioveta, CSSR, has been available since 1981 and consists of two inactivated strains of SVCV. The vaccine is administered in an oil emulsion by in-

traperitoneal injection. A live vaccine has been developed by Fijan *et al.* (1977) who passed the virus on human diploid, BHK-21, and FHM cells. The live vaccine has been administered by intraperitoneal inoculation, bath immersion and by feeding. There are drawbacks to both of these vaccines. The inactivated preparation lacked potency at low temperature and the live vaccine was only partially attenuated (Fijan, 1988).

Prospects for a subunit vaccine for the carp rhabdoviruses should be good. The viral glycoprotein gene for SVCV has been cloned and the development of a subunit vaccine for SVCV should be possible (Roy *et al.*, 1984).

Infectious pancreatic necrosis virus has been found in fish species around the world. It has been isolated from a number of salmonid fish species in North and South America, Europe, and Asia. It has also been found to be pathogenic in eels, loach, cichlids, cyprinids, yellowtail, and menhaden. Because of its widespread presence among cultured fish, killed and attenuated vaccines for IPNV have been under investigation for a number of years (Dorson, 1988). However, immunization with formalin inactivated virus appeared to be effective only after administration by injection. Oral immunization or hyperosmotic infiltration of the killed virus preparation did not confer any protection. Several investigators have tried to develop an attenuated IPNV vaccine but as soon as a virus had lost pathogenicity for fry, it appeared to lose immunogenicity (Dorson, 1988).

More recently, several laboratories have begun developing subunit vaccines for IPNV by recombinant DNA techniques (Leong *et al.*, 1988). The viral genome has been cloned and the major capsid protein, VP2, has been expressed in bacteria for several IPNV strains. The bacterially expressed protein was shown to be effective in inducing protective immunity in rainbow trout fry (Manning, 1988).

An attenuated vaccine has been developed for *Herpesvirus ictaluri* (Noga & Hartmann, 1981). The attenuated virus was produced after repeated passage in walking catfish kidney cells and found to be very effective after immersion vaccination of young fish. The vaccine did prevent death in fish exposed to otherwise lethal doses of virulent virus; however, it was not determined whether the attenuated or challenge virus produced a carrier infection in the catfish. Since herpesviruses are well known for forming latent infections, this is a question which should be considered by vaccine producers.

For the fish pox herpesvirus which attacks valuable adult fish (ornamental carp), a vaccine would be acceptable and economically viable. The virus produces epithelial tumors that are aesthetically undesirable in either ornamental or table fish. Yet, there have been no reports of vaccine development for this viral agent.

In addition to the fish viruses, there are the viruses that cause severe epizootics among cultured prawns (Lightner, 1985). Production of *Penaeus* sp. (marine prawns) can be curtailed by oubreaks of *Baculovirus monodon* or Infectious Hypodermal and Hematopoietic Necrosis Virus. Because the immune response to protein antigens is not well developed in crustaceans, vaccines for shrimp have not been considered seriously by many investigators. Yet, killed bacterins have been effective in controlling Gaffkaemia in lobsters (Paterson, 1989) and vibriosis in shrimps (S-N. Chen, personal communication). It is possible that viral vaccines may be developed for cultured shellfish.

Prospects for Bacterial Fish Vaccines

As mentioned previously, there are several highly successful vaccines against

the more important fish pathogens, but a number of difficult problems remain. Notable among these are the need for improved vaccines against furunculosis and bacterial kidney disease (BKD), which remain major disease problems, especially in marine culture of salmonids.

Although several commercial furunculosis vaccines are widely used, there does not seem to be universal agreement about their efficacy in all situations. Many workers have investigated the properties of extracellular products of *Aeromonas salmonicida* such as proteases and haemolysins (Ellis *et al.*, 1981; Titball & Munn, 1985; Fyffe *et al.*, 1988) and their potential use as vaccines (Hastings, 1988), but the complex nature of crude ECP, and the inactivation of protective antigens, seem to be particular problems. The role of individual components of ECP in pathogenicity has been the subject of much controversy, and it is likely that several components show synergistic effects (Titball & Munn, 1985; Lee & Ellis, 1989). Recently, the major lethal toxin of *A. salmonicida* has been identified as glycerophopholipid: cholesterol acyltransferase (GCAT) complexed with lipopolysaccharide (LPS; Lee & Ellis, 1989) and this is an obvious candidate for investigation as a vaccine. The activity can be purified using FPLC ion-exchange chromatography (Lee & Ellis, 1989) but the problem of ensuring inactivation without impairing immunogenicity could remain a problem. The genes encoding GCAT and proteolytic activity have recently been cloned (Gilpin & Munn, 1989), and the possibility of gene manipulation giving rise to polypeptides with altered properties now exists. However, it is important to note the fact that GCAT is naturally complexed with LPS, both in *A. salmonicida* and in recombinant *E. coli*, and its potential immunoprotective properties remain to be established. At least, cloning of the individual ECP components will allow some of the questions about their role in pathogenicity to be determined. One other obvious potential candidate for inclusion in a subunit vaccine is the regularly-structured surface array (A-protein) which has been demonstrated to be important in resistance to host defence mechanisms (Munn *et al.*, 1982; Trust *et al.*, 1983) and in determining attachment to epithelial cells (Parker & Munn, 1985). The A-protein has now been cloned by Belland & Trust (1987), but further progress seems to have been hampered by their inability to obtain stable subclones. Similar difficulties have been experienced in work at Plymouth (M L Gilpin, personal communication). Also, the role of A-protein as a protective antigen is by no means certain (Austin & Austin, 1987), and controversy surrounds the assumption that it is an essential requirement for virulence. An alternative approach to the use of subunit vaccines is the development of an attenuated live vaccine. Vaughan and Foster (Trinity College Dublin, Ireland) have successfully used gene cloning techniques to produce aromutants which appear to be fully attenuated, with no evidence of replication or survival in fish after use of very high inocula (P R Smith, personal communication). Initial studies of the immune response of fish to these mutant bacteria are very promising, and controlled field trials are being planned to confirm that protection is conferred. It is worth noting that Cipriano & Starliper (1982) obtained good results with an attenuated *A. salmonicida* vaccine produced by conventional means.

In the case of BKD, caused by *Renibacterium salmoninarum*, there seems to be little hope of producing an effective vaccine by conventional means. The major problem here lies in the fact that the bacterium grows extremely slowly in culture, and hence it is very difficult to ensure a stable and reproducible supply of ECP or surface antigens for investigation as potential vaccine candidates. Large-scale commercial production of a vaccine

from a bacterium with such inherent problems of culture seems very unlikely. Recently, a gene encoding the production of *R.salmoninarum* haemolysin has been cloned in *E. coli* (Evenden *et al.*, 1990), and the immunological properties of the cloned product are now being investigated. It is likely that similar successes will be achieved shortly with other important antigens such as the surface antigen (haemagglutinin).

In addition to these examples, there are many situations where improved vaccines would be beneficial, and recombinant DNA technology may be useful. For example, gene cloning methods have been helpful in the elucidation of the detailed molecular mechanisms underlying the pathogenicity of *Vibrio anguillarum* (Crosa, 1989). At present, commercially-produced *Vibrio* vaccines incorporate both *V. anguillarum* and *V. ordalii* (Smith, 1988). The situation is complicated in regions where vaccination against 'cold-water vibriosis' caused by *V. salmonicida* is important. Also, there is growing recognition of strain variation among vibrios isolated from different geographical regions, and it is questionable whether bacterins based on one or two isolates will give universal protection as they become employed in different aquaculture locations. Identification of protective epitopes common to diverse strains might lead to new vaccines.

Finally, in theory at least, there exists the possibility of producing 'cocktail' vaccines which would stimulate immunity against several diseases simultaneously, thus avoiding the need for fish farmers to administer several vaccines. A possible way forward might be to develop shuttle vectors which allow transfer of genes between the various fish pathogens, so that a bacterium for which there is a well-established delivery technology (such as *Vibrio*) could be used as a carrier of several different antigens. However, it may be that antigenic competition will present insuperable difficulties to the use of this approach in fish.

Prospects for Vaccines Against Parasitic Fish Diseases

The salmon louse (*Lepeophtheirus salmonis*) is currently causing significant losses, especially in Scottish and Norwegian aquaculture (Pike, 1989). The parasite cannot be cultured axenically, so the prospects for vaccine development look bleak. However, if antigens from larval stages of the parasite could be identified, the possibility exists of cloning these in bacteria, yeast or some other expression system. Several groups are currently attempting to isolate antigens from the parasite and demonstrate potential as protective antigens, and work is also being undertaken to construct gene libraries of the parasite. However, production of a vaccine is dependent on improved knowledge of transmission and pathogenicity and is likely to be several years away.

Another strong candidate for the application of new molecular techniques is white spot, caused by *Ichthyophthiriasis multifiliis*. Protection has been achieved using cilia from related ciliates of the genus Tetrahymena and this may be used as a basis for future vaccines (Houghton *et al.*, 1988). Gene cloning could be used to isolate and produce antigens in quantity.

Licensing and Environmental Considerations

In most countries, the use of recombinant DNA technology is regulated by government agencies, and legal obligations must be fulfilled. When it comes to the use of products such as subunit vaccines produced by cloning in *E. coli* or yeast, it is unlikely that there will be any reasonable environmental objections, and licensing should be straightforward. The host organism will be killed before

delivery, or the antigen will be extracted and purified, and there should be no problems provided the usual quality assurance criteria of sterility and safety to fish are documented. For example, the U.S. Department of Agriculture has approved field trials of the IHNV vaccine described above.

However, the use of live attenuated vaccines in aquaculture is highly controversial. In general, most authorities consider live vaccines to be undesirable because of the risk of uncontrolled spread in the aquatic environment. In some cases, such as VHS, the severity of the disease problem has led fish farmers to put pressure on government laboratories to use a live vaccine (produced by conventional methods) as the only alternative to an eradication programme. The possibility of reversion is considered by some to be an insignificant problem when large numbers of the virulent pathogen are already present (P de Kinkelin, personal communication), but there are possible dangers. For example, a virus strain shown to be non- virulent for the particular farmed species of fish may well be highly pathogenic for other species in the natural ecosystem. The environmental consequences of use of live vaccines will require careful consideration and would require extensive research. When the additional complication of the use of recombinant DNA technology is introduced, additional proof of safety and environmental compatibility is likely to be needed for live vaccines since the environmental release of genetically-manipulated organisms is highly regulated. In Europe and North America, it seems that licensing authorities are at present very unlikely to approve such vaccines and future development does not look promising.

Economic Considerations and Conclusions

Although offering great promise, the potential use of recombinant DNA technology in vaccine development can, unfortunately, be criticised from the viewpoint that the unit cost of vaccines developed by this means will be too high to be economically viable. The general view among vaccine manufacturers is that the aquaculture industry would not be prepared to pay a realistic price for a vaccine which would allow the recovery of research costs. It is true that much of the basic research can be very costly, and in many cases it can be argued that this can only properly be financed by government agencies (as in the case of IHNV). However, once this development work is done, the costs of testing and licensing a recombinant subunit vaccine are no more than for a conventional method, and in many cases will be much lower because complex purification steps may not be needed. In spite of the pessimistic view expressed by vaccine manufacturers about the economic viability of developing recombinant vaccines, we consider that it is important that basic research is maintained, especially in those situations which are threatening the very survival of the industry and where there is little hope of developing vaccines along traditional lines.

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Note:
reference to EAFP abstracts refers to the Conference Handbook, EAFP IV International Conference, Santiago de Compostela, Spain, September 1989.

Summary

Recombinant DNA technology or gene manipulation can be defined as the formation of new combinations of genetic material by the insertion of nucleic acid molecules, produced outside the cell, via a vector system such as bacterial plasmid, virus, or cosmid. This allows the insertion and continued propagation of genetic material into a host organism in which it does not naturally occur. The implications of this technology are, of course, very far-reaching. On one hand, it allows the manipulation of genes from unrelated organisms, and the crossing of species barriers which would not be possible by conventional genetic methods. Secondly, it allows the isolation and production of pure DNA fragments in quantities which permits the determination of sequences, and modifications and manipulation to ensure the high expression in the host organism of polypeptides encoded by the DNA.

In this article, we examine the background to the use of recombinant DNA technology in the development of vaccines. Considerable advances have been made in the development of new human and veterinary vaccines and by showing examples of these successful applications we illustrate the various approaches possible. We then discuss progress to date in the successful production of a recombinant vaccine for the control of an important virus disease of fish, and consider the prospects for using genetic manipulation in the development of vaccines for other virus, bacterial and parasitic diseases of fish. Finally, we address a series of practical questions. Although recombinant DNA technology opens up many theoretical pos sibilities, there are some very important economic, environmental and legal aspects which must be considered.

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MANUSCRIPT # 12

Recombinant Viral Vaccines in Aquaculture

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Recombinant Viral Vaccines in Aquaculture*

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ABSTRACT

Viral pathogens in aquaculture have largely been controlled by the culling and destruction of carriers and infected animals and eggs. Because most viral pathogens in aquaculture are transmitted via water and because sensitive animals reside in the neighboring waters, the administration of attenuated viral vaccines has not been feasible. Attenuated vaccines require costly trials to assure that these modified live viruses are nonvirulent in all species and that reversion to virulence does not occur. Killed viral vaccines have been too expensive to produce for the aquaculture industry. Thus, subunit viral vaccines developed by recombinant DNA techniques are attractive alternatives for the industry. These vaccines are nonreplicating and inexpensive to produce. The molecular cloning and expression of viral genes in several host vector systems for the development of subunit viral vaccines for aquaculture has been the primary research focus of the authors' laboratory. Work on the development of such vaccines for infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, and infectious pancreatic necrosis virus (IPNV), a fish birnavirus, is presented. Laboratory tests of both vaccines *in vivo* have indicated that fish develop protective immunity to live virus after vaccination.

Introduction

One of the major factors that will have an impact on the success of the aquaculture industry is the control of diseases. As the industry grows and greater productivity demands are made on facilities, the incidence of disease outbreaks will increase. Thus, the need for more effective disease controls has been receiving more attention. The viral diseases are particularly important because there are no suitable treatments available. In the United States, there are no approved antiviral drugs or vaccines that can be used in the aquaculture industry today.

At the present time, the control of viral diseases is based largely on management. Current recommendations for the control of viral disease outbreak include the destruction of diseased stocks, drainage of ponds, disinfection of contaminated areas with chlorine, treatment with sunlight or lime, and the restocking of the facility with disease-free stock. These procedures are very expensive and instituted with understandable reluctance by the industry. With earthen ponds and stable viruses, like the baculoviruses and the picornaviruses, these disinfection procedures may not work.

Another facet of present day controls for viral diseases in aquaculture is the requirement for certified pathogen-free stocks and eggs and the use of specific pathogen-free water in the facility. When available, these requirements have been very effective in preventing disease outbreaks. However, it has not always been possible to obtain disease-free stocks for highly-prized strains nor economically practical to use specific pathogen-free water. Thus, the aquaculture industry has a definite need for viral vaccines. Our group reports here the successful development of two prototype viral vaccines by recombinant DNA techniques.

Two viruses were selected for vaccine development because these viruses affect economically important aquaculture species (salmon and trout) in the United States, Europe, and Japan. In addition, these viruses, infectious hematopoetic necrosis virus (IHNV), and infectious pancreatic necrosis virus (IPNV), affect very young fish, and immunization of large numbers of fish at this size by immersion is fairly easy. IHNV is a rhabdovirus with an enveloped virion and glycoprotein peplomers on the

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envelope surface; it has a viral genome of single-stranded RNA of negative sense (McAllister and Wagner 1977). In contrast, IPNV is a nonenveloped virus with a single capsid and a genome of two segments of double-stranded RNA (Dobos 1975). The techniques that were involved in the construction of recombinant plasmids containing the genes for the major immunogenic proteins of these two viruses have been described (Kurath and Leong 1985; Huang et al. 1986). The expression of these proteins in bacteria and the use of these expressed proteins as vaccines will be described here.

Materials and Methods

Cells, Virus, and Antisera

The following viruses here used in this study: the IHNV isolate from Round Butte was obtained from W. Groberg (Oregon Department of Fisheries and Wildlife) and the IPNV isolates, Sp and Buhl, were obtained from R. Hedrick, University of California at Davis. The virus used for challenge studies was prepared by infecting rainbow trout (*Oncorhynchus mykiss*) fry and reisolating the virus from fish dying of IHN disease in the case of the IHNV isolates and IPN disease in the case of the IPNV isolates. Subsequently, the virus was grown for two passages in chinook salmon embryo cells (CHSE-214 cells) (Fryer et al. 1978). The tissue-culture supernatant fluid containing the virus was used as the challenge virus. The IHNV and IPNV used as molecular weight markers in Figure 1 were prepared as described in Kurath and Leong (1985) for IHNV and Huang et al. (1986) for IPNV. The rabbit antisera prepared against purified IHNV and IPNV were made as described (Engelking and Leong 1989).

Construction of Recombinant Plasmids

The construction of a recombinant plasmid containing the *trp* E promoter and the *trp* E gene fused to an immunogenic region of the gene for IHNV glycoprotein gene or the IPNV VP2 gene is shown schematically in Figure 2. The isolation, cloning and sequence analysis of these genes have been reported (Koener et al. 1987; D. S. Manning 1988). The pATH vectors were the generous gift of A. Tzagaloff (Dieckmann and Tzagoloff 1985). The constructions were verified by DNA sequence analysis by the dideoxy method (Sanger et al. 1977). The plasmid pUC 19, which served as the negative control for pTA1 in Figure 1 was obtained from Pharmacia, Inc., Piscataway, NJ.

Immunization Trials in Fish

Bacterial crude lysates were prepared as described (Kleid et al. 1981). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting as previously described (Gilmore et al. 1988). The crude lysates were used to immunize fish by immersion. Rainbow trout fry at 0.4 g were immunized in sets of 100 fish. Immunization was accomplished by bathing groups of 100 fry in 25 mL of the vaccine preparation (ca. 3 mg/mL total protein concentration) for 1 minute. At that time, the immersion solution volume was increased to 250 mL with water and fish were incubated in this diluted solution for an additional

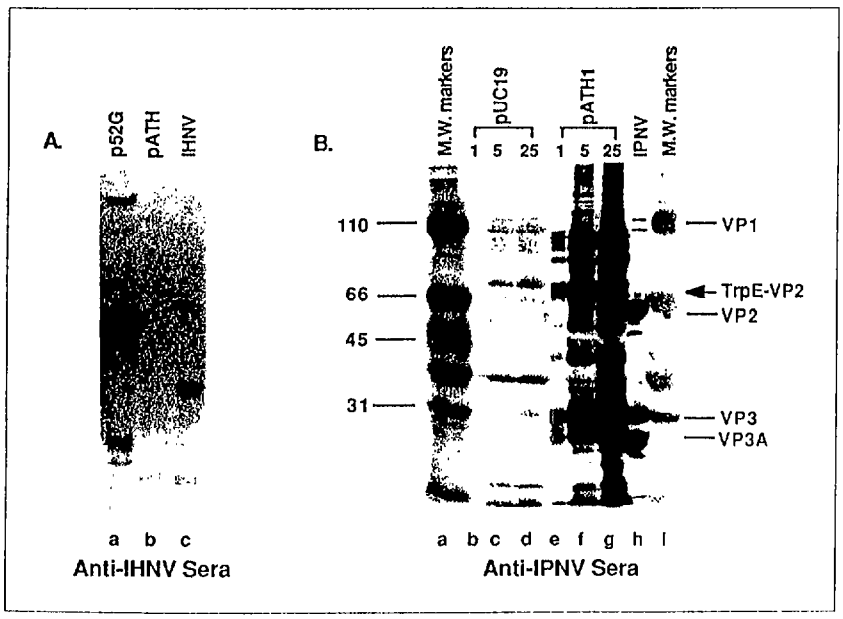

Figure 1

Analysis of bacterial production of *trp* E-viral gene fusion proteins by antibody reactivity on an electrophorectic transfer blot of a 10% SDS-polyacrylamide gel of bacterial extracts. (A) Development of the blot made with anti-IHNV sera. Lane a is the *trp* E-G fusion protein detected in cells containing the recombinant plasmid, p52G; Lane b are proteins detected in cells containing the expression vector pATH3, without a viral gene insert; and Lane c is purified IHNV. (B) Development of the blot made with anti-IPNV sera. Lanes a and i contain the molecular weight marker proteins: phosphorylase B (110000 Da); bovine serum albumin (66000 Da); ovalbumin (45000 Da); and carbonic anhydrase (31000 Da). In lanes b, c, and d are cell lysates from bacterial cells containing the plasmid pUC19 with no viral insert; the samples were loaded at 1, 5 and 25 μL respectively in lanes b, c, and d. In lanes e,

2 minutes. These fish were then placed in aquaria of 5 gallons with a water flow rate of 0.25 gal/min in a constant water temperature of 10°C. The control fish were exposed to saline in the same procedure or left undisturbed.

Approximately one month after immunization, the experimental and control fish were placed in separate aquaria in groups of 25. The fish were exposed to serial log virus dilutions in 1 liter of water. The challenge virus was prepared as described by Engelking and Leong (1981). In Figure 3, the data for fish exposed to $7.2 \times 10^{+5}$ plaque forming units per mL (PFU/mL) is presented. The data represents the mean of duplicate experiments. All dead fish were assayed for the presence of infectious virus in chinook salmon embryo cells (CHSE-214) as described by Engelking and Leong (1981).

Results

Antigen Production in Bacteria

The size and quantity of virus-specific antigen produced in bacteria hosting the recombinant plasmids was estimated from stained gels and Western blots of total bacterial extract. In Figure 1, the product of a *trp*E-IHNV glycoprotein fusion gene from the plasmid p52G and the major capsid protein of IPNV from the plasmid pTA1 is shown in Western blots of the appropriate bacterial lysates. A determination of the DNA sequence of p52G indicated that a 264 bp fragment of the IHNV glycoprotein gene had been inserted in-frame with the *trp*E protein to produce a fusion protein of 49000 daltons (49 kDa = 37 kDa [*trp*E] + 11 kDa [glycoprotein gene fragment]). In addition, a second fragment of the IHNV glycoprotein gene had been inserted out-of-frame adjacent to the 264 bp fragment and this additional nucleic acid resulted in 1 kDa (84 bp extra) more of amino acid residue owing to the fusion protein (Gilmore et al. 1988).

The IPNV expressing plasmid, pTA1, contained the entire coding region of the A segment of the viral genome for the isolate Sp. It was constructed so that the viral genetic information was fused in-frame to the *trp*E protein (Figure 2) and all the proteins encoded by the A segment were synthesized in the bacteria. Thus, VP2 (major capsid protein), and VP3 (minor capsid protein)

Figure 1 (Continued)
f, and g are 1, 5 and 25 μL of cell lysates from bacterial cells containing the plasmid pTA1. Lane h contains purified IPNV. The arrow indicates the *trp*E-VP2 fusion protein found in lanes e, f, and g. The symbol VP1 indicates virion protein 1; VP2, virion protein 2; VP3, virion protein 3; and VP3a, breakdown product of VP3.

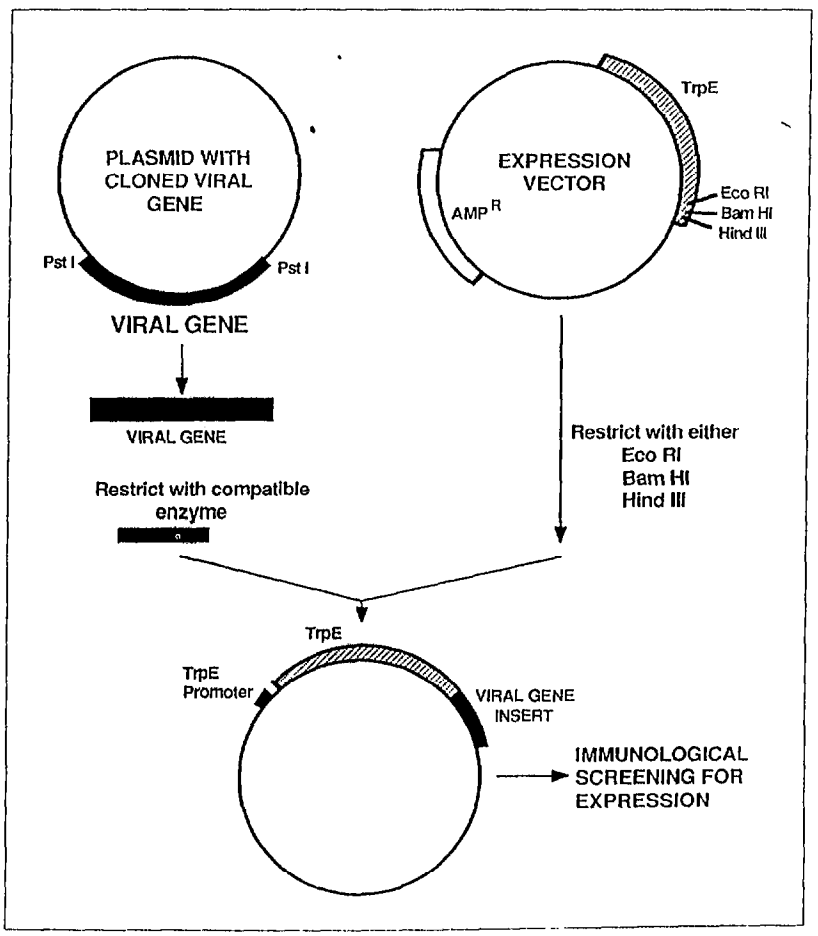

Figure 2
Construction of the expression vectors for *trp*E-viral gene fusions. The cDNA cloned insert of the IHNV glycoprotein gene or the A segment of the IPNV genome was restricted with a compatible nuclease to permit the insertion of a portion of the viral gene into the expression vector in the proper reading frame. The resulting plasmids were used for expression of a *trp*E-viral gene fusion protein in *E. coli*.

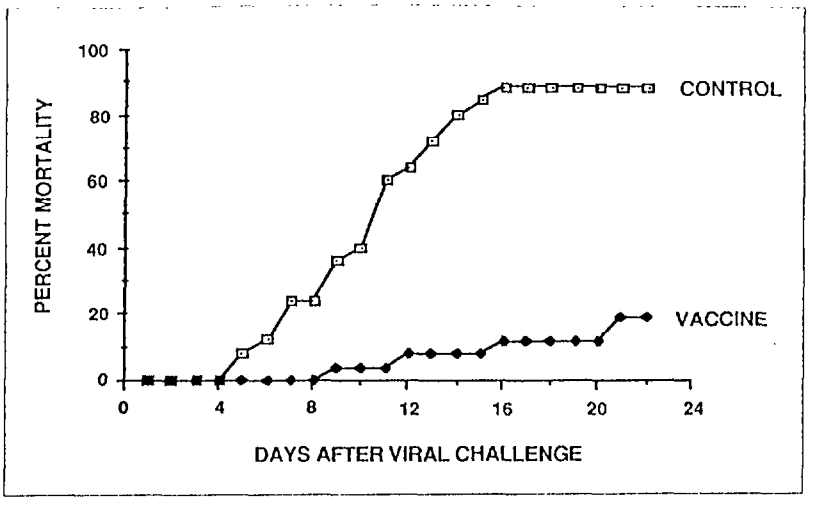

Figure 3
Immunization of rainbow trout with a subunit vaccine against IHNV. Rainbow trout fry (0.4 g) were immersed in a bacterial lysate (3 mg/mL, containing 10% expressed *trp*E-G fusion protein) as described in Materials and Methods section. The results are expressed as mean percent total mortality on the ordinate and days after the initiation of viral challenge on the abscissa. There were 25 fish in the control group and 26 fish in the vaccinated group. In this particular challenge, the fish received $7.2 \times 10^{+5}$ plaque forming units/mL.

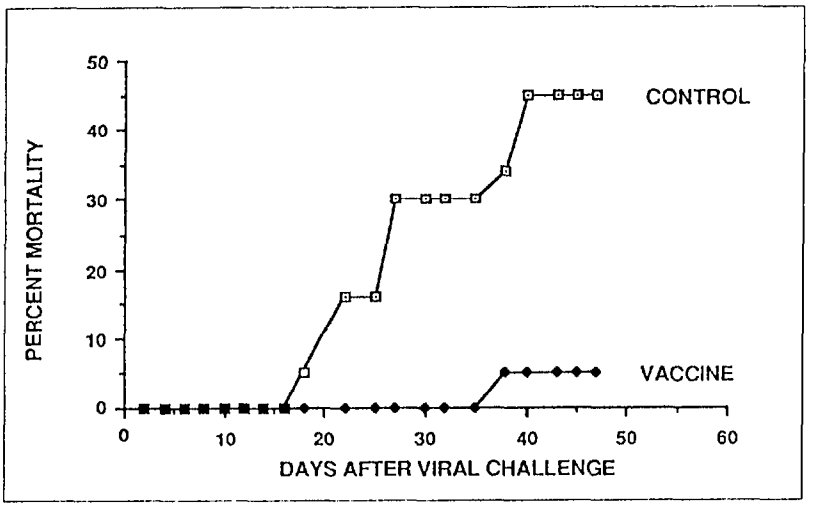

Figure 4
Immunization of rainbow trout with a subunit vaccine against IPNV. Rainbow trout fry (0.3 g) were immersed in a bacterial lysate (3 mg/mL) containing the *trp*E-VP2 fusion protein as described in Materials and Methods section. The results are expressed as percent total mortality on the ordinate and days after the initiation of viral challenge on the abscissa. There were 25 fish in both control and vaccinated groups. The fish received 10^{+6} plaque forming units/mL of IPNV-Buhl strain for viral challenge.

of IPNV-Sp were expressed by this recombinant plasmid in bacteria.

Immunization Trials with Subunit Vaccine

Viral challenges provided data on the efficacy of the bacterially expressed protein as vaccines. A significant level of protection (69%) was conferred on fish immunized with p52G versus unimmunized fish when challenged with the Round Butte isolate of IHNV (Figure 3). The glycoprotein used in constructing the fusion protein was derived from this strain. In Figure 4, the protection that was achieved by immunization with pTA1 against the Buhl isolate of IPNV, a heterologous virus strain, is shown. A decrease in virus-induced mortalities from 45% to 3% was found for the immunized group of fish.

Discussion

We have presented initial findings on the efficacy of bacterially expressed viral proteins as subunit vaccines for fish. Both the IHNV and the IPNV vaccines were effective in immunizing fish against lethal viral challenge in laboratory trials. Moreover, the vaccinations were carried out on rainbow trout fry that were 0.4 g in size. These fish were able to respond effectively to the viral vaccine. Previous studies of immunization in fish have indicated that the minimum size for successful immunization by immersion was 0.8 g for chinook salmon (Fryer et al. 1978) and 1–2.5 g for rainbow trout (Johnson et al. 1982).

The use of these vaccines with different species of fish and against a variety of different viral strains must be tested. In addition, the duration of effective immunity must be determined. However, the possibility now exists for

developing an inexpensive and effective vaccine for fish using recombinant DNA technology.

The development of any vaccine must have safety as well as efficacy as one of its primary considerations. The safety of live attenuated vaccines has been questioned for the aquaculture industry because of the nature of the environment where the vaccine would be applied. The vaccine has to be completely safe for cultured and wild salmonid fish in the watershed. Moreover, the vaccine has to be economical and a subunit vaccine produced in bacteria seems to be a viable alternative. The initial trials of the subunit vaccines reported here suggest that bacterially expressed viral proteins, even in crude lysates, can be used as effective and economical viral vaccines.

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MANUSCRIPT # 13

Epitope Mapping and Characterization of the Infectious Hematopoietic Necrosis Virus Glycoprotein, Using Fusion Proteins Synthesized in *Escherichia coli*

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Epitope Mapping and Characterization of the Infectious Hematopoietic Necrosis Virus Glycoprotein, Using Fusion Proteins Synthesized in *Escherichia coli*†

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A characterization of the antigenic determinants (epitopes) of the glycoprotein (G) of infectious hematopoietic necrosis virus was made by expressing different regions of the G gene in *Escherichia coli*. A cDNA copy of the G gene was divided into four fragments by *Taq*I digestion, and the fragments were subcloned into pATH vectors, placing the expression of each G gene fragment under control of the *trpE* promoter. The resulting plasmids, pXL2, pXL3, and pXL7, encoded *trpE*-G fusion proteins subsequently detected with anti-infectious hematopoietic necrosis virus sera by Western immunoblots. A comparison of reactivities of the fusion proteins encoded by these plasmids was made by Western immunoblot and radioimmunoassay with a number of anti-G specific monoclonal antibodies (MAbs). The nonneutralizing MAb 136J reacted with the *trpE*-G fusion protein encoded by pXL3 and fusion proteins encoded by plasmids p52G and p618G, which were described in previous studies (R. D. Gilmore, Jr., H. M. Engelking, D. S. Manning, and J. C. Leong. Bio/Technology 6:295–300, 1988). Another nonneutralizing MAb, 2F, bound to the pXL3 fusion protein, and the neutralizing MAb RB/B5 recognized the pXL7 fusion protein. All fusion proteins were tested as vaccines in rainbow trout fry. Although significant protection was induced by all fusion proteins, the pXL3 fusion protein was most effective as a vaccine.

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus associated with severe epizootic disease in juvenile salmonid fish (17, 23). IHNV infections have resulted in large economic losses to salmon and trout industries. Presently, there are no chemotherapeutic agents or licensed vaccines available for prevention or control of the disease; however, upon immunization with killed IHNV, fish produce protective serum antibody which neutralizes IHNV in vitro (1). Because attenuated and killed vaccines are extremely expensive and have not proven totally efficacious (2, 13), the aquaculture industry is looking for advances in recombinant DNA technology to provide economical and effective vaccines.

The IHNV virion contains an unsegmented negative-sense single-stranded RNA genome of approximately 11,000 nucleotides encoding five structural proteins and one nonvirion protein (11, 15, 16). The glycoprotein, found on the surface of IHNV, has been identified as the single viral protein which induces the production of neutralizing antibodies in both rabbits and fish. With the glycoprotein, cross-protective immunity in fish against challenge with different electrophoretic types of IHNV is obtained (5). It therefore may be possible to develop an economical subunit vaccine for controlling the disease patterned after the glycoprotein of a single type of IHNV. Polyvalent rabbit antiserum raised against enzymatically deglycosylated virus retains virus-neutralizing activity and reacts with glycosylated G protein in Western immunoblots (5), indicating that different types of IHNV share a neutralizing epitope(s) not involving carbohydrate side chains.

Previous work with rabies virus glycoprotein showed that expression of the full-length glycoprotein gene in bacteria resulted in a denatured protein unable to produce a protective immune response, presumably because inappropriate folding mechanisms for disulfide bond formation existed in *Escherichia coli* (14). Thus, we sought smaller portions of the IHNV glycoprotein gene coding for one or a few epitopes, instead of the intact gene, for construction of recombinant plasmids for bacterial expression (6).

The IHNV glycoprotein gene of 1,609 nucleotides encoding a protein of 508 amino acids has been cloned and sequenced (10). Two recombinant plasmids, p52G and p618G, containing approximately 350 bases of the *Sau*3AI-digested IHNV G gene, have been constructed and expressed in bacteria as *trpE*-G fusion proteins. The two plasmids contain identical G gene fragments of 329 bp encoding amino acids (aa) 336 to 444, which were ligated in frame to the *trpE* protein, but they differ by additional G gene sequences which were ligated out of frame to the 3′ ends of the 329-bp fragments (6). The molecular masses of the fusion proteins expressed by p52G and p618G were 49 and 48 kDa, respectively. Bacterial lysates containing *trpE*-G fusion proteins made from p52G or p618G induced protective immunity against IHNV in laboratory trials (6) and led to the development of a prototype IHNV subunit vaccine (6). Despite the efficacy of the vaccine in laboratory trials, there still remained questions concerning the number of immunoprotective regions on the G protein and the identification of those regions recognized in fish. These questions led to the development of a number of expression clones containing different regions of the G protein. The construction of clones and the characterization of the epitopes of their expressed fusion proteins are described

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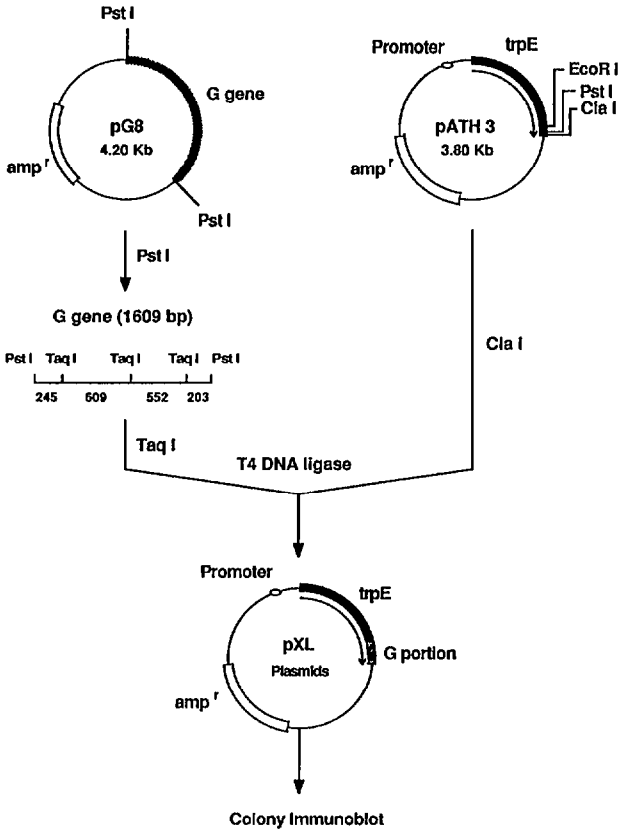

FIG. 1. Construction of *trpE*-G fusion protein expression plasmids. The 1.6-kb cDNA of the IHNV G gene isolated from the pG8 plasmid was digested with *Taq*I. The G gene fragments were purified and ligated to *Cla*I-digested pATH3.

here in an analysis of the antigenic nature of the IHNV G protein.

Portions of the G gene were subcloned into *trpE* expression vectors to create *trpE*-G fusion proteins (Fig. 1). *Taq*I digestion of a 1.6-kb *Pst*I fragment from pG8, which contained the entire G gene, resulted in four fragments: A (245 bp), B (609 bp), C (552 bp), and D (203 bp). Recombinant plasmids were constructed by the ligation of purified preparations of each fragment or of a mixture of all four fragments with the pATH3 vector which had been previously digested with *Cla*I. The ampicillin-resistant transformant colonies were screened for IHNV G protein expression by colony immunoblot (7) with rabbit polyclonal anti-IHNV sera. Three plasmids (pXL2, pXL3, and pXL7) isolated from three positive colonies were chosen for subsequent analyses. Direct plasmid DNA sequence analyses (8, 21) verified that pXL2 contained the *Taq*I B fragment and pXL3 contained the *Taq*I C fragment (Fig. 2). The pXL7 plasmid, which was derived from the ligation mixture of all four fragments, contained the 3′ external D fragment ligated in frame to the *trpE* gene. In addition, the 5′ external A fragment was ligated to the 3′ external fragment at the *Pst*I site in the same orientation as the G gene (Fig. 2).

Fusion proteins were expressed in *E. coli* DH5α cells after indoleacrylic acid induction as previously described (6). The induced bacterial cells were harvested by centrifugation and lysed by high-speed agitation in a minibeadbeater (Biospec Products) with Tris-EDTA buffer (10 mM Tris–1 mM EDTA, pH 8.0). The *trpE*-G fusion proteins encoded by pXL2, pXL3, and pXL7 were detected by Western immunoblot (12, 20) with anti-IHNV serum, and the estimated molecular masses of each protein were 59.3, 57.3, and 43 kDa, respectively (Fig. 3). The *trpE* component of the fusion protein contributed 340 aa (37,400 kDa) to each fusion protein. These estimates were consistent with the molecular masses calculated from the derived amino acid sequence obtained from the plasmid DNA sequence analyses for pXL2 (aa 66 to 269), pXL3 (aa 270 to 453), and pXL7 (aa 454 to 508).

The location of linear antigenic determinants of the IHNV G protein were then determined by reaction with monoclonal antibodies (MAbs) on Western immunoblots (20). These results were compared with those for the proteins produced by p52G (aa 336 to 444) and p618G (aa 336 to 444). The study included 10 MAbs which had been shown to be reactive with the IHNV glycoprotein (2, 3). Only those MAbs which reacted with the IHNV G protein in Western immunoblots are described here. The nonneutralizing MAb 136J bound to the pXL3 (aa 270 to 453), p52G (aa 336 to 444), and p618G (aa 336 to 444) fusion proteins (Fig. 4A). The nonneutralizing MAb 2F reacted only with the pXL3 (aa 270 to 453) fusion protein (Fig. 4B). The pXL7 (aa 454 to 508) fusion protein was recognized by neutralizing MAb RB/B5 (provided by J. L. Fryer, Oregon State University [22]) (Fig. 4C). Table 1 summarizes the binding activity of each MAb to the *trpE*-G fusion proteins.

The pXL2 (aa 66 to 269) plasmid encoding a *trpE*-G fusion protein was recognized by anti-IHNV serum (Fig. 3) but not by any of the anti-G MAbs. Although there are two external *Taq*I-digested G gene fragments (totaling 510 nucleotides) inserted into pXL7 (aa 454 to 508), the *Taq*I D fragment was the only sequence expressed as part of the *trpE*-G fusion protein. Examination of the sequence indicated that there was a TAA termination codon at the end of the coding sequence in the *Taq*I D fragment (position 1547 in the original glycoprotein gene) and no consensus recognition site for translational initiation (19) of the *Taq*I A fragment sequence. Therefore, a peptide of only 55 aa from the G gene was encoded by pXL7 (aa 454 to 508). This peptide reacted with both anti-IHNV serum (Fig. 3) and the neutralizing MAb RB/B5 (Fig. 4C). The results indicated that there was a neutralizing epitope recognized by RB/B5 within this C-terminal region of 55 aa. A hydropathy plot of the amino acid sequence of the IHNV G protein indicated that a very large hydrophobic domain, presumably the transmembrane region, was encoded in the G fragment cloned into the pXL7 (aa 454 to 508) plasmid. This hydrophobic domain consists of 27 aa from 461 to 487. This would suggest that the

TABLE 1. Summary of the immunoblot characterization of G subunits by MAbs

IHNV isolate[a]	MAb	Ig type[b]	Neutralizing ability[c]	Reactivity of plasmids tested by Western blot assay[d]				
				pXL2	pXL3	pXL7	p52G	p618G
039-82-SR	136J	IgG	–	–	+	–	+	+
Cedar River	2F	IgM	–	–	+	–	–	–
Round Butte	RB/B5	IgM	+	–	–	+	–	–

[a] IHNV isolates that were used to generate the MAbs.
[b] Ig, Immunoglobulin.
[c] –, Unable to neutralize; +, able to neutralize.
[d] +, Reactivity; –, no reactivity.

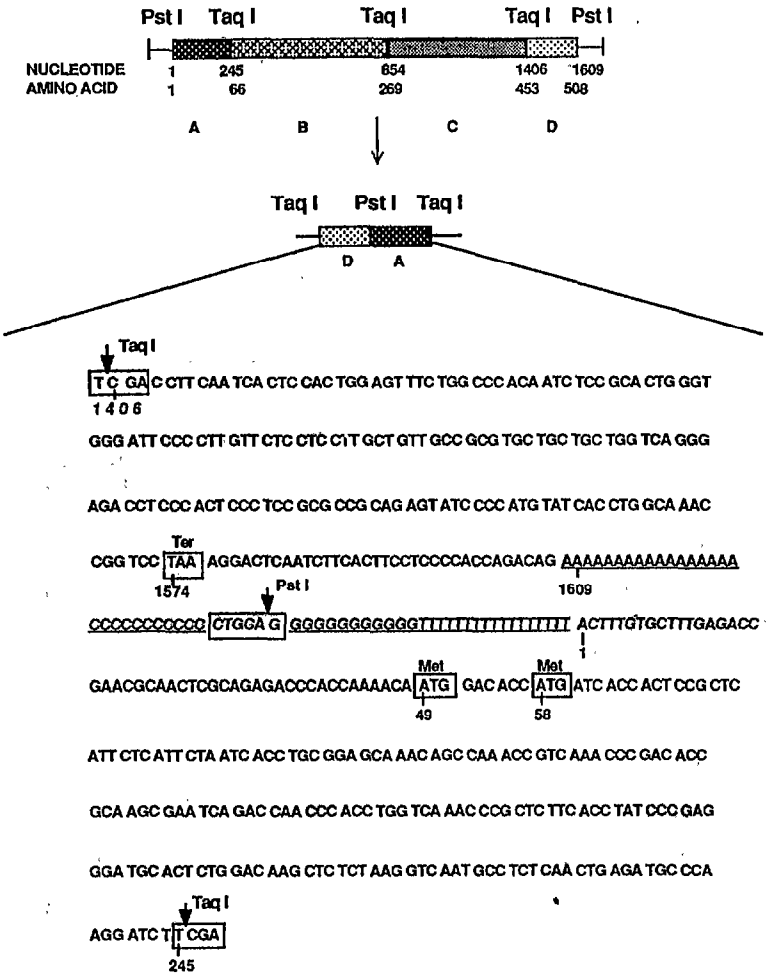

FIG. 2. DNA sequence of the cloned G gene fragments in the plasmid pXL7.

RB/B5 epitope is localized to the N-terminal region (aa 453 to 460) of the G fusion protein encoded by pXL7 (aa 454 to 508).

Although the plasmids pXL3 (aa 270 to 453), p52G (aa 336 to 444), and p618G (aa 336 to 444) had DNA inserts of different sizes, they all contained a common G gene sequence from nucleotides 1052 to 1379 (Fig. 5). The plasmid pXL3 (aa 270 to 453) contained an additional 198 nucleotides of the G gene sequence at the 5′ end. These plasmids expressed *trpE*-G fusion proteins of 57.3, 49, and 48 kDa, respectively. All of these proteins reacted with polyclonal anti-IHNV sera (Fig. 3) and the nonneutralizing MAb 136J (Fig. 4A) in Western immunoblots. This result indicated that the common 109-aa sequence (336 to 444) encoded by these three plasmids contained a linear epitope recognized by MAb 136J.

The MAb 2F recognized by Western immunoblot a unique epitope which was found only in the G subunit defined by pXL3 (aa 270 to 453) (Fig. 4B). This result indicated that there was a distinct linear epitope, 2F, located in the unique 66 aa at the N terminus of the pXL3 (aa 270 to 453)-encoded G protein sequence. MAb 2F was generated against the Cedar River strain of IHNV, while MAb 136J was raised against the 039-82-SR strain of IHNV. Since pXL3, a clone containing G gene sequences from the Round Butte strain of

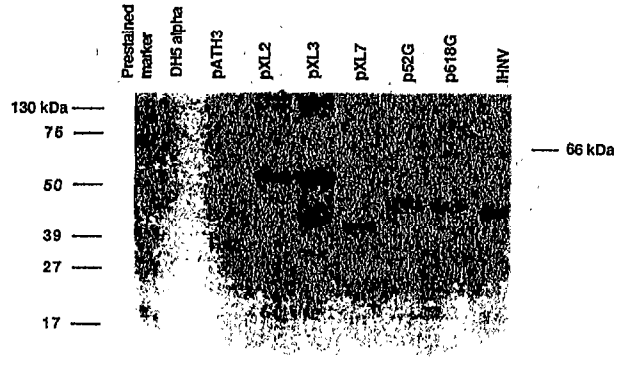

FIG. 3. Analysis of *trpE*-G fusion proteins. Bacterial lysates were resolved on sodium dodecyl sulfate–10% polyacrylamide gels. The proteins on the gels were transferred to nitrocellulose and analyzed with anti-IHNV serum. The *trpE*-G fusion proteins are shown. The major bands expressed by pXL2, pXL3, pXL7, p52G, and p618G migrated at positions of 59.3, 57.3, 43, 49, and 48 kDa, respectively.

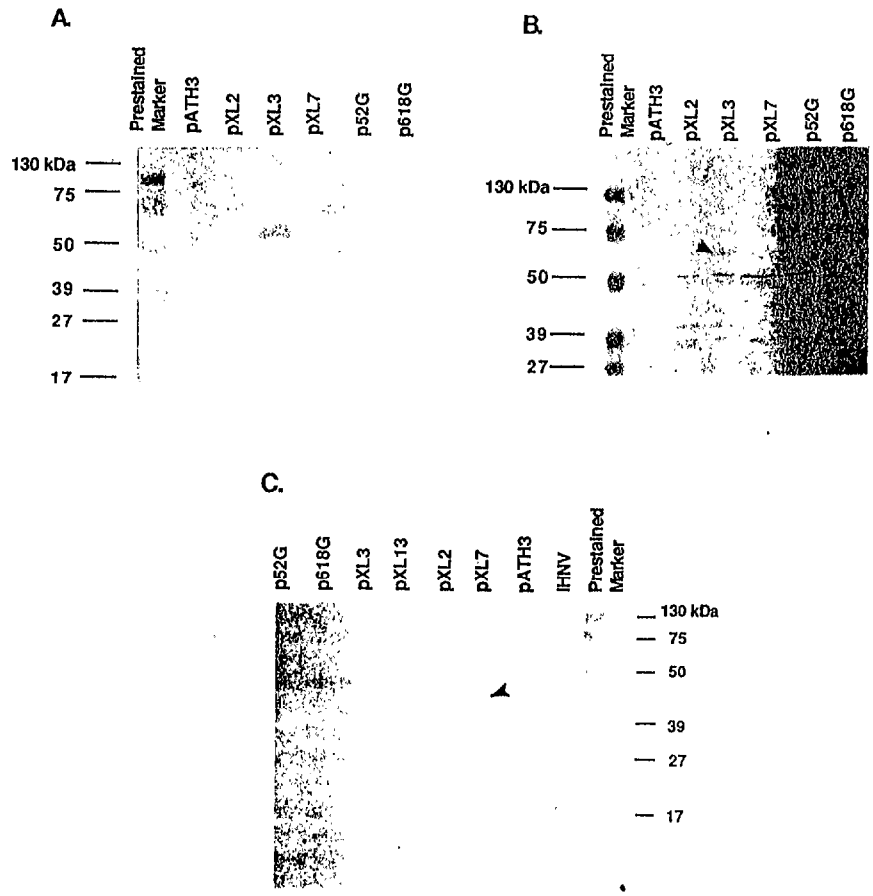

FIG. 4. Immunoblot characterization of the *trpE*-G fusion proteins with MAbs. Bacterial lysates were resolved on sodium dodecyl sulfate–10% polyacrylamide gels, and the proteins were transferred to nitrocellulose. Each blot was developed with the MAb indicated below. Each lane is marked at the top by the plasmid used to generate the protein samples that were added to that gel well. The location of the prestained molecular mass marker is shown on the side. (A) MAb 136J. The pXL3 (57.3-kDa), p52G (49-kDa), and p618G (48-kDa) fusion proteins were recognized by 136J. (B) MAb 2F. The pXL3 fusion protein (57.3 kDa) is indicated by the arrowhead. (C) MAb RB/B5. The pXL7 fusion protein (43 kDa) is indicated by the arrowhead. The band running just above the fusion protein band is a common cross-reactive polypeptide that is found in the bacterial lysates. The IHNV lane does not have any reactive bands because too little viral protein was used in this gel. However, RB/B5 does react with the glycoprotein of IHNV in immunoblots.

IHNV, reacted with the two MAbs, 136J and 2F, all three virus strains must contain common epitopes defined by 2F and 136J (Fig. 4A and B). A diagrammatic representation of these results is shown in Fig. 5.

Lysates of induced cultures of pXL2 (aa 66 to 269), pXL3 (aa 270 to 453), p52G (aa 336 to 444), and pXL7 (aa 454 to 508) were used for the vaccination of 100 rainbow trout fry (0.4 g each) by immersion. Thirty days after the vaccination, the fry were challenged with 100 and 1,000 50% tissue culture infective doses of the Box Canyon IHNV per ml (a strain which has been classified as a type 2 isolate) (5). Moribund fish were collected on a daily basis for 30 days. The presence of virus in all these fish was confirmed by the production of characteristic cytopathic changes in tissue

TABLE 2. Relative protection induced in fish by bacterial lysates with different plasmids

Experimental group	% Mortality with virus challenge dose[a]	
	100	1,000
Control	56.0	75.0
pXL2	26.0	48.0
pXL3	2.5	14.0
p52G	19.0	56.0
pXL7	36.0	50.0

[a] Average percent mortality in three replicate samples of 30 fish each. The virus challenge dose is the number of 50% tissue culture infective doses per ml as described by Gilmore et al. (6).

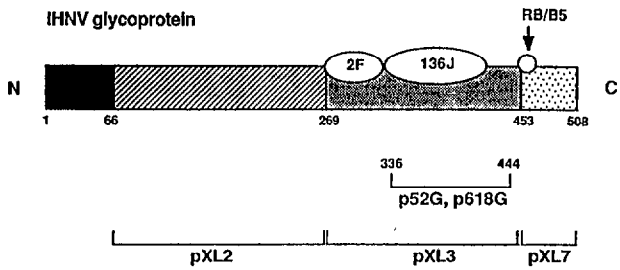

FIG. 5. Epitope map of the IHNV glycoprotein.

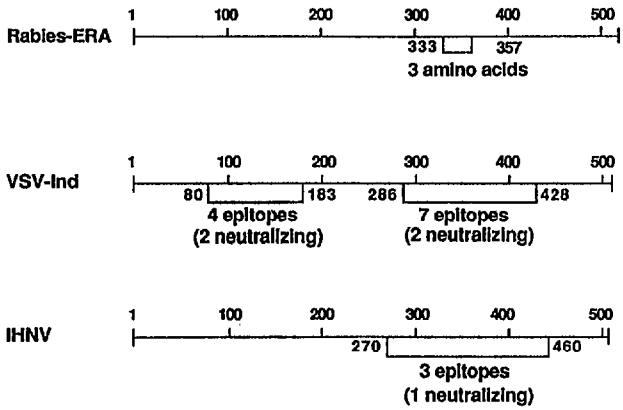

FIG. 6. Comparison of the locations of epitopes on the glycoproteins of IHNV, vesicular stomatitis virus (VSV-Ind), and rabies virus (rabies-ERA).

culture. Table 2 shows the average percent mortalities in each experimental group. The pXL3 (aa 270 to 453)-encoded region of the IHNV glycoprotein induced a strong immunoprotective response in fish. Although the regions encoded by pXL2 (aa 66 to 269), p52G (aa 336 to 444), and pXL7 (aa 454 to 508) also induced some protection, the pXL3 (aa 270 to 453) lysate was clearly much more protective as a vaccine.

Although there is a very low level of protein sequence homology between the IHNV glycoprotein and the glycoproteins of two other animal rhabdoviruses, vesicular stomatitis virus and rabies virus, the structural domains on these three glycoproteins (transmembrane region, signal peptide, positions of cysteines, and location of glycosylation sites) have also been conserved (10). It should also be possible to determine whether there is conservation among the antigenic structures of the rhabdovirus glycoproteins. The seven epitopes of the Indiana serotype of vesicular stomatitis virus, including two neutralizing epitopes, have been mapped to the amino acid sequence 286 to 428 (Fig. 6) (9). For the rabies virus (Evelyn-Rokitnicki-Abelseth strain) glycoprotein, amino acids Arg-333, Asn-336, and Gly-357 were found to be essential for neutralization (4, 18, 24). We have localized three epitopes of the IHNV glycoprotein in the region of aa 270 to 460 (Fig. 4A and B).

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MANUSCRIPT # 14

Comparison of Representative Strains of Infectious Hematopoietic Necrosis Virus by Serological Neutralization and Cross-Protection Assays

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Comparison of Representative Strains of Infectious Hematopoietic Necrosis Virus by Serological Neutralization and Cross-Protection Assays†

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Infectious hematopoietic necrosis virus (IHNV) is a pathogen of young salmon and trout. Viral epizootics among these fish in private and public rearing facilities have been a problem in the northwestern United States from California to Alaska, and an IHNV vaccine has been sought by the aquaculture experts. Since an IHNV vaccine must be designed to immunize against all viral serotypes, an analysis of IHNV serotypes was made. A large number of viruses from widely separated geographic locations and different fish species had already been placed in one of five electropherotypes by the migration of the virion proteins in sodium dodecyl sulfate-polyacrylamide gels. Also, there was evidence that some of these virus isolates had differences in virulence for chinook salmon, rainbow trout, or kokanee salmon. Previous serological studies with polyclonal rabbit antisera and three IHNV isolates indicated that there was only one serotype (B. B. McCain, J. L. Fryer, and K. S. Pilcher, Proc. Soc. Exp. Biol. Med. 137:1042–1046, 1971). A substantial number of new IHNV isolations have been made since that study, and thus a more extensive comparison was made of 10 different IHNV isolates representing the five electropherotypes. This report shows that the glycoprotein from a single isolate of IHNV can induce a protective immune response in vivo to the five IHNV electropherotypes. Plaque reduction neutralization assays indicated that there was only one serotype. Thus, despite the differences observed in the migration of the structural proteins for IHNV isolated from separate geographic locations and different fish species, only one neutralizing virus type was identified.

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that produces an acute disease in salmon and trout resulting from the destruction of the hematopoietic tissue in the kidneys. The only effective means for controlling this disease is the complete destruction of stocks of infected fish and sterilization of the hatchery. These control methods are expensive and may lead to the loss of the entire hatchery production (23). It is clear that a vaccine for IHNV is needed by both public and private fish culturists.

We had shown previously that an analysis of the virion protein patterns of 71 isolates of IHNV led to the classification of these virus isolates into five different types (12). This initial classification provided information that was useful in determining that a particular virus type was characteristic for a geographic area and would infect many different salmonid species in that area. Also, it provided a means for determining whether a new virus type had been introduced into the area so that effective fish management decisions could be made about the movement and shipment of eggs and fish from the affected site. However, the need for an IHNV vaccine to control the spread of this disease in the environment has made it important to determine whether there is also a biological basis for distinguishing these five electropherotypes.

The IHNV glycoprotein had been shown to be the only viral protein capable of eliciting a neutralizing antibody response in rabbits and providing protective immunity in young fish (8). These experiments showed that immunization of rainbow trout (*Oncorhynchus mykiss*) and kokanee fry (*O. nerka*) with purified glycoprotein (G protein) from IHNV Round Butte strain (RB1) did protect the fish against challenge with a potentially lethal IHNV infection (8). The work was extended to show that the purified G protein from RB1 also induced protective immunity against the five IHNV types by immersion vaccination (9). This work suggested that there was only one serotype of IHNV. Also, early work (21) and current diagnostic tests (3) suggested that there was a single serotype of IHNV. However, differences in levels of neutralization indicating strain variation were noted by these workers. Winton et al. (27) separated 12 IHNV strains into four groups by use of three monoclonal antibodies. Differences in virulence of three IHNV strains have also been observed (19).

In the study reported here, an effort was made to determine whether there was any correlation between electropherotypes and reactivity to polyclonal rabbit anti-IHNV sera in virus neutralization assays. Also, a comparison of the effectiveness of immersion versus inoculation vaccination of purified G protein was made for the five electropherotypes to determine whether immersion vaccination would be effective in vaccine development.

MATERIALS AND METHODS

Cells and virus. Chinook salmon (*O. tshawytscha*) embryo cells, CHSE-214, were grown in RPMI 1640 medium supplemented with fetal calf serum (5%), penicillin (100 IU/ml), and streptomycin (100 μg/ml) as previously described (10). Virus assays were done with confluent CHSE-214 cell monolayers grown in 24-well tissue culture plates (Falcon). Samples from infected fish were prepared as described previously (3), sterilized by filtration (0.2-μm-pore-size Acrodisc; Gelman),

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and diluted in minimal essential medium without fetal calf serum. Duplicate samples (0.1 ml) of each dilution were placed on monolayers in individual wells and allowed to adsorb for 60 min. After this period, sample inocula were removed from the wells and 1.0 ml of minimal essential growth medium was added to each well.

The RB1 (type 1) and the Elk River (ER, type 3) strains of IHNV were obtained from W. Groberg, Oregon Department of Fish and Wildlife. The Hagerman Valley (HA1, type 2) strain was obtained from N. Wood, Rangen Research Laboratories, Buhl, Idaho. The Coleman River National Fish Hatchery (CO2, type 4) and Cedar River (CD2, type 5) strains were obtained from D. Mulcahy, National Fisheries Research Center, Seattle, Wash. The viral G protein was isolated from RB1 after extensive purification of the virus by isopycnic and velocity sedimentation centrifugation as described below. All virus strains were prepared by growing the virus at a multiplicity of infection of 0.01 to 0.001 50% tissue culture infective dose ($TCID_{50}$) per cell on CHSE-214 cells as previously described (10). The virus used for challenges in the immunization trials was prepared from a stock of virus which had been passaged no more than three times in tissue culture after its isolation from infected fish.

Virus purification for antisera. The virus isolates RB1 and CO2 were propagated in CHSE-214 cells grown in 175-cm^2 plastic tissue culture flasks (Falcon). The cell monolayers were infected at a multiplicity of infection of 0.001 PFU per cell and incubated at 16°C for 7 days or until the monolayers showed complete cytopathic effect. The supernatant fluid was harvested, and cell debris was removed by centrifugation at 3,000 × *g* for 10 min at 4°C in a Sorvall HS-4 rotor. The virus lysate was pelleted onto a 0.3-ml pad of 100% glycerol by using a Beckman SW28 rotor for 60 min at 90,000 × *g*. The virus pellet was resuspended in 0.01 M Tris buffer (pH 7.5). A 10-ml sample of this virus suspension was centrifuged through a discontinuous gradient composed of 7 ml of 50% sucrose–0.01 M Tris (pH 7.5), 10 ml of 35% sucrose–0.01 M Tris (pH 7.5), and 10 ml of 20% sucrose–0.01 M Tris (pH 7.5) for 90 min at 90,000 × *g* with an SW28 rotor. The virus band was collected from the interface between the 20 and 35% sucrose layers. This band was concentrated by centrifugation in 0.01 M Tris (pH 7.5) at 90,000 × *g* for 1 h. The pellet was resuspended in 0.01 M Tris (pH 7.5) and banded in a continuous gradient of 5% sucrose–0.01 M Tris (pH 7.5) and 30% sucrose–0.01 M Tris (pH 7.5) in an SW41 rotor at 49,000 × *g* for 30 min. The virus band was collected and centrifuged in an SW41 rotor for 1 h at 100,000 × *g* and 4°C. The resulting virus pellet was resuspended in 0.5 ml of 0.01 M Tris (pH 7.5) and stored at −70°C.

Antiserum preparation. Antisera to purified virus or specific viral proteins were prepared in 3-month-old New Zealand White male rabbits. A series of three immunization doses were administered by intradermal injection at multiple sites. The first immunization dose contained either 250 μg of IHNV or 100 μg of specific protein antigen mixed 1:1 (vol/vol) with complete Freund adjuvant. The second and third immunizations were given 2 and 4 weeks later, respectively. These booster injections contained one-half the amount of antigen used in the initial injection, and the antigen was mixed 1:1 (vol/vol) with incomplete Freund adjuvant. Blood samples were drawn from the animals at 7 to 10 days after the last immunization episode. Those rabbits identified as hyperimmune by enzyme-linked immunoassay were bled by cardiac puncture. The antiserum was heat inactivated for 30 min at 56°C, and aliquots of the serum were stored at −70°C.

Neutralization assays. Plaque assays were performed as described by Burke and Mulcahy (5). Serial 10-fold dilutions of virus were incubated with equal volumes of various dilutions of antiserum at 15°C for 3 h on a rotation shaker. Duplicate wells in a six-well plate (Falcon) containing monolayer cultures of CHSE-214 cells were inoculated with 0.2 ml of each antiserum-virus mixture. After 1 h at 15°C, the infected cells were overlaid with 0.8% gum tragacanth (Fisher Scientific Co.) in minimal essential medium with 5% fetal calf serum. After 7 to 10 days at 15°C, the cells were fixed, stained, and counted. The relative virus titers with and without antiserum treatment were determined and used to calculate the plaque reduction endpoint dilutions. All determinations were repeated in three separate experiments with duplicate measurements for each assay. Neutralization indices (n.i.) were calculated by the method of Casals (6) and used to compare the neutralizing titers of the antisera.

Purification and analysis of viral G protein. The IHNV G protein was purified as previously described (20). Briefly, purified IHNV was incubated in 1% Triton X-100 to selectively solubilize the G protein. The other viral proteins were removed by centrifugation. The Triton X-100 in the G protein preparation was removed by batch elution with SM2 beads (Bio-Rad).

Electrophoresis was performed in a 10% polyacrylamide gel with a 3% stacker as described by Laemmli (18). Protein bands were visualized by staining the gel with Coomassie brilliant blue or silver nitrate as described by Allen (1).

Vaccination trials in fish. The fish utilized in these studies were obtained from Oregon Department of Fish and Wildlife hatcheries through R. Holt, Oregon State Fish Pathologist. The fry, weighing about 0.3 g per fish (1 to 2 months posthatching), were obtained 1 week before immunization and kept at the Fish Disease Laboratory in Corvallis, Oreg., to reduce the possible effects of the stress of the transport on the immunization process. The fry were maintained on BioDiet (BioProducts, Warrenton, Oreg.) in well water flowing at 0.5 gallons (ca. 1.9 liters)/min (1 tank volume per h) at 12°C. When the fish were 0.4 g in size, they were vaccinated by direct immersion or intraperitoneal inoculation.

The fish were first anesthetized with benzocaine, and then each fish was injected intraperitoneally with 10 μl of purified IHNV G protein from a 250-μl glass syringe with a 30-gauge disposable needle. The inoculated fish received 0.4 to 0.5 μg of purified G protein. Immersion immunization was performed on 100 nonanesthetized fish in a 200-ml beaker containing a total volume of 4 ml of purified G protein (40 to 60 μg/ml in 0.01 M Tris, pH 7.6). The fish were exposed to the protein solution for 2 min and then released into a holding tank containing running water at 12°C. Control fish were mock immunized by immersion in 0.01 M Tris (pH 7.6) alone.

Challenges were made in lots of 25 vaccinated and unvaccinated control fish for each virus dilution (14, 15). The fish were exposed to serial log virus dilutions in 1 liter of water. The method of Reed and Muench (24) was used to determine the $TCID_{50}$ titer of the challenge virus. The fish were held in the virus-containing water for 18 h and then returned to holding tanks. Dead fish were removed from each tank, weighed, processed for IHNV isolation, and tallied on a daily basis (3). For those samples in which IHNV-induced cytopathic effect was questionable, the tissue culture fluid from the sample well was removed and reinoculated onto CHSE-214 cells and subsequently labeled with [^{35}S]methio-

TABLE 1. Description of IHNV variants used in cross-challenge protection studies and cross-neutralization tests

Type[a]	Location	Fish[b]	Age or tissue	Date of isolation	Designation
1	Round Butte Hatchery, Oreg.	StS	Ovarian	1975	RB1
1	Tamgas Creek, Alaska	Pink	Juvenile	1981	TA1
2	Hagerman, Idaho	RbT	Adult	1978	HA1
2	Lewis River, Wash.	Ch	Juvenile	1980	LE
3	Elk River, Oreg.	ChF	Fry	1979	ER
3	Dworshak National Fish Hatchery, Idaho	StS	Yearling	1980	DW2
3	Dworshak National Fish Hatchery, Idaho	StS	Juvenile	1985	DW3
3	Nan Scott Lake, Oreg.	RbT	Juvenile	1971	NS
4	Coleman National Fish Hatchery, Calif.	Ch	Alevin	1980	CO2
5	Cedar River, Wash.	ChF	Ovarian	1981	CD2

[a] See Table 2.
[b] Abbreviations: Ch, chinook salmon; ChF, fall run chinook salmon; RbT, rainbow trout; StS, summer steelhead trout.

nine as described previously for gel analysis of viral proteins (13).

Only those fish from which IHNV was isolated were considered positive in these studies. The percent mortality was determined for each group, and the relative percent survival (RPS) was calculated for each group of vaccinated fish compared with infected, mock-immunized controls as described by Johnson et al. (15): RPS = [1 − (% specific loss vaccinated/% specific loss controls)] × 100.

The data were analyzed by logit regression. The number of fish that died from IHNV infection was taken to be a binomial random variable with the probability of death, P, depending on the various factors under investigation. The amount of virus required to produce a lethal dose for 50% of the infected fish (LD_{50}) was determined for the vaccinated and control groups by the method of Reed and Muench (24). When an LD_{50} was not achieved, an estimate of the LD_{50} titer was made by extrapolation.

RESULTS

Comparative protection of G protein-immunized fish. A critical question in the development of any viral vaccine is whether cross-immunity will arise from immunization with a vaccine derived from a single virus strain. To determine whether cross-protection was possible, we immunized fish by intraperitoneal injection or immersion with G protein isolated from IHNV type 1 Round Butte (RB1) and challenged them with various isolates representing the other four biochemical types (Table 1). The virus doses used in the challenges were substantially higher than those described in the natural aquatic environments (22).

The biochemical IHNV types have been defined by differences in the electrophoretic mobilities of the nucleocapsid protein (N protein) and G protein (12) (Table 2). Isolates of IHNV that were used as heterologous virus in the challenge study were chosen because they represented the IHNV types found in the Columbia River Basin, an area where IHNV is enzootic (Table 1). The type 4 isolate, CO2, was included because this isolate is virulent in chinook salmon (6a) and has a G protein of greater apparent molecular weight by electrophoretic mobility (12).

TABLE 2. Protein molecular weight characteristics of the types of IHNV

Virus protein	Mol wt (10^3)				
	Type 1	Type 2	Type 3	Type 4	Type 5[a]
N	40.5	42.8	43.25	40.5–41.0	41.0–44.0
G	67.0	67.0	67.0	70.0	67.0

[a] Type 5 is less defined and N protein varies in these isolates (12).

The type 2 strains were represented by the Hagerman, Idaho, IHNV isolate (HA1) from the Snake River valley. LaPatra et al. (19) have shown this IHNV strain to be more virulent for rainbow trout than the representative type 1 or 3 IHNV isolate. The protection induced in fish immunized by inoculation or immersion vaccination with G protein from RB1 (type 1) and challenged with HA1 (type 2) is shown in Table 3. Statistical analysis of the data indicated that protection against endogenous levels of the heterologous type 2 strain of IHNV (10^{-4} and 10^{-5} dilutions) was induced by the type 1 G protein. At epizootic levels of IHNV HA1 (10^{-2} and 10^{-3} dilutions) protection was minimal. The LD_{50} for the mock-immunized group was 4.2×10^2 $TCID_{50}$/ml, indicating a relatively virulent IHNV strain.

An Elk River, Oreg., IHNV isolate, ER, represented the type 3 IHNV strain. Type 3 virus isolates were also found at the Dworshak National Fish Hatchery, Idaho, and this virus type may be important in the upper Columbia River region. This isolate is commonly a pathogen of chinook salmon. Virulence of the type 3 IHNV strains is similar to that of type 1 (19). Rainbow trout were immunized with the type 1 purified G protein and challenged with Elk River type 3 IHNV. Immunization was very effective in protecting fish against the type 3 virus challenge (Table 3). The level of protection that was achieved was similar to that of the homologous type 1 IHNV challenge (8). The LD_{50} of 5×10^3 $TCID_{50}$/ml for the control group indicated a less virulent IHNV strain than the HA1 isolate.

The type 4 IHNV isolates are temperature-sensitive viral variants which were obtained from chinook salmon at the Coleman National Fish Hatchery (12, 22). When the immunized fish were challenged with the biologically characterized IHNV isolate, CO2, good protection was noted for fish vaccinated by immersion or injection (Table 3). Similar results were obtained when the vaccinated fish were challenged with CD2 IHNV, a type 5 virus isolated from sockeye salmon in the Cedar River, Wash. (Table 3). The LD_{50}s (Table 3) for the control groups are similar to those reported by others (2, 6a). To produce an LD_{50} in vaccinated fish, more virus would be required (17 to 428 times more, depending on the strain of IHNV). These results indicate that immunization with the G protein from a type 1 IHNV isolate by either intraperitoneal inoculation or immersion will induce protective immunity to heterologous virus isolates.

TABLE 3. Comparison of survival, after challenge with IHNV types 2 to 5, of kokanee salmon and rainbow trout fry immunized by inoculation or immersion with the G protein of IHNV type 1[a]

Log virus dilution	Inoculated				Immersed				Control		
	No.[b]	SL[c]	%[d]	RPS[e]	No.	SL	%	RPS	No.	SL	%
(A) Challenge with HA1 in kokanee salmon (type 2)											
−2	24	10	42	51	25	8	32	62	20	17	85
−3	12	8	62	13	23	9	39	45	17	12	71
−4	9	1	11	82	5	3	60	0	25	15	60
−5	20	5	25	50	15	6	40	20	24	12	50
LD_{50}[f]	3.8×10^4				6.9×10^3				2.2×10^3		
(B) Challenge with HA1 in rainbow trout (type 2)											
−2	9	6	67	33	11	10	91	9	12	12	100
−3	10	4	40	56	11	7	64	30	12	11	92
−4	9	2	22	71	10	4	40	47	12	9	75
LD_{50}	1.8×10^5				4.2×10^3				4.2×10^2		
(C) Challenge with ER in rainbow trout (type 3)											
−2	25	4	16	75	25	1	4	94	25	16	64
−3	25	0	0	100	25	3	12	80	25	15	60
−4	25	0	0	100	25	0	0	100	25	8	32
−5	25	0	0	100	25	0	0	100	25	1	4
LD_{50}	$>2.3 \times 10^5$				$>2.3 \times 10^5$				5.0×10^3		
(D) Challenge with CO2 in rainbow trout (type 4)											
−2	25	8	32	57	25	8	32	57	20	15	75
−3	25	4	16	80	25	5	20	75	20	16	80
−4	25	2	8	84	25	2	8	84	20	10	50
−5	25	0	0	100	25	0	0	100	20	5	25
LD_{50}	$>5.0 \times 10^5$				$>5.0 \times 10^5$				5.0×10^3		
(E) Challenge with CD2 in rainbow trout (type 5)											
−2	22	9	41	59	25	10	40	56	25	25	100
−3	22	7	32	67	25	9	36	63	25	24	96
−4	21	4	19	75	25	7	28	63	25	19	76
−5	22	0	0	100	25	3	12	70	25	10	40
LD_{50}	4.6×10^5				4.6×10^5				2.4×10^3		

[a] The titer of the stock virus for each virus strain was as follows: HA1 in trial A = 2.2×10^8 $TCID_{50}$/ml; HA1 in trial B = 4.2×10^7 $TCID_{50}$/ml; ER = 1.0×10^8 $TCID_{50}$/ml; CD2 = 4.6×10^7 $TCID_{50}$/ml; CO2 = 5.0×10^7 $TCID_{50}$/ml.
[b] No., number of fish in group.
[c] SL, specific loss, i.e., number of fish dying from IHNV infection.
[d] %, percent mortality.
[e] RPS, relative percent survival (15).
[f] LD_{50}, lethal dose of IHNV in $TCID_{50}$ per milliliter to cause 50% mortality.

Serological comparison of IHNV strains by cross-neutralization assays. The cross-neutralization analyses were done with antisera prepared to purified IHNV RB1 and IHNV CO2 and to the purified G proteins for the two virus types. The type 4 isolate, CO2, was the only virus isolate that contained a G protein whose molecular weight was different (Table 2).

When the n.i. obtained for anti-IHNV RB1 serum were compared with that obtained for anti-IHNV CO2 (Table 4), at least two different virus groups were distinguished. The comparisons were made between the results obtained at a 1:100 dilution of anti-IHNV RB1 serum and a 1:20 dilution of the anti-IHNV CO2 serum because these dilutions gave approximately the same level of neutralization for the virus isolates RB1 and CO2. One group contained viruses which were neutralized significantly (n.i. > 1.7) by both antisera (RB1, TA1, HA1, ER, CO2, CD2). A second group, containing NS, LE, DW2, and DW3, was neutralized to a lesser degree or not at all. A similar grouping of the virus isolates was recognized by the anti-G protein serum as well (Table 5). An analysis of the n.i. obtained with a 1:40 dilution of the anti-IHNV RB1 G protein serum indicated that this dilution produced the same approximate level of neutralization as the 1:100 dilution of the anti-IHNV RB1 serum. Two groups of viruses were recognized: one contained the viruses more sensitive to the anti-IHNV RB1 G protein serum (RB1, TA1,

TABLE 4. n.i.[a] for 10 IHNV isolates tested with antisera to IHNV RB1 and CO2

Isolate (type)	n.i.					
	Anti-IHNV RB1 at a dilution of:			Anti-IHNV CO2 at a dilution of:		
	1:50	1:100	1:200	1:5	1:20	1:50
RB1 (1)	C[b]	2.4	1.0	ND[c]	1.8	0.6
TA1 (1)	2.6	2.0	0.3	ND	2.5	1.9
HA1 (2)	C	2.1	0.3	ND	2.4	1.5
LE (2)	1.5	0.9	0.1	0.20	0	0
ER (3)	3.0	2.0	0.5	ND	3.7	1.5
NS (3)	1.4	1.1	0	2.8	1.5	0
DW2 (3)	1.5	0.5	0	1.6	0.2	0
DW3 (3)	1.7	0.4	0	1.7	0.1	0
CO2 (4)	C	1.8	0.7	2.4	1.8	0.2
CD2 (5)	C	C	1.5	2.8	2.6	0.8

[a] n.i. (6) = $\log_{10}$[(PFU of control virus/ml)/(PFU of antiserum-treated virus/ml)]. n.i. ≥ 1.7 is significant neutralization (98% neutralization). n.i. = 1.0 to 1.7 is intermediate neutralization (>90% neutralization). n.i. = 0.3 to 0.9 is questionable neutralization (>50% neutralization). n.i. < 0.3 is no neutralization (<50% neutralization).

[b] C, complete neutralization, no viral titer, so an index cannot be computed.

[c] ND, not done because of high n.i. at 1:20 antiserum dilution.

ER, and HA1); the other was defined by virus more resistant to this antiserum (LE, NS, DW2, DW3, CO2, and CD2).

By using antiserum directed against the G protein of IHNV CO2, two groups of similar members again emerged. At a 1:100 dilution of this antiserum, six isolates (RB1, TA1, ER, HA1, CO2, and CD2) were significantly neutralized (Table 5). NS gave an intermediate n.i., and the three other isolates were not neutralized. At a 1:50 dilution of this antiserum, only DW3 exhibited some reduction in titer. Again, LE, DW2, and DW3 were found to be antigenically distinct from the other virus isolates. NS appeared to occupy a position between the strongly and weakly neutralized groups. The one aberrant observation in this set of neutralizations (Table 5) was the result obtained with the CO2 isolate, which was weakly neutralized by anti-IHNV CO2

TABLE 5. n.i.[a] for 10 IHNV isolates tested with antisera to the G proteins of IHNV RB1 and CO2

Isolate (type)	n.i.				
	Anti-G protein of IHNV RB1 at a dilution of:		Anti-G protein of IHNV CO2 at a dilution of:		
	1:20	1:40	1:50	1:100	1:200
RB1 (1)	4.9	2.0	C	C	1.6
TA1 (1)	C[b]	1.9	C	C	2.73
HA1 (2)	2.8	1.7	C	2.1	1.3
LE (2)	C	1.6	0.1	ND[c]	ND
ER (3)	3.2	2.0	3.7	C	2.5
NS (3)	1.8	0	C	0.6	0
DW2 (3)	3.5	1.0	0.2	0	ND
DW3 (3)	2.4	0.5	0.4	0	0
CO2 (4)	C	1.1	C	3.6	1.7
CD2 (5)	2.9	1.2	C	2.7	1.2

[a] See Table 4, footnote *a*.

[b] C, complete neutralization, no viral titer, so an index cannot be computed.

[c] ND, not done because of the low n.i. at the previous dilution.

TABLE 6. Cross-neutralization antigenic relatedness test (4)

Specific antiserum	Immune serum	Heterologous virus strain	Titer ratio[a]
Whole virus[b]	Anti-IHNV RB1	CO2	0.8 (r_1)
	Anti-IHNV CO2	RB1	0.9 (r_2)
G protein[c]	Anti-IHNV RB1 G protein	CO2	0.4 (r_1)
	Anti-IHNV CO2 G protein	RB1	1.33 (r_2)
	Anti-IHNV RB1 G protein (electroeluted)	CO2	0.75 (r_3)

[a] Titer ratio for whole virus = (dilution of antiserum neutralizing 90% of heterologous virus)/(dilution of antiserum neutralizing 90% of homologous virus). Titer ratio for G protein = (dilution of antiserum neutralizing 98% of heterologous virus)/(dilution of antiserum neutralizing 98% of homologous virus).

[b] $r = \sqrt{r_1 \times r_2} = 0.72$; antigenic relatedness value = $1/r = 1.38$, or 73% related.

[c] $r = \sqrt{r_1 \times r_2} = 0.73$; antigenic relatedness value = $1/r = 1.37$, or 73% related. $r = \sqrt{r_1 \times r_3} = 1.00$; antigenic relatedness value = $1/r = 1.00$, or 100% related, where $1/r = 1$ is identity and $1/r = 2$ is 50% related.

serum and significantly neutralized by the anti-IHNV CO2 G protein serum.

In summary, a comparison of the n.i. with four different antisera led to the identification of a virus group (LE, DW2, DW3, and NS) that was weakly neutralized and another group (RB1, TA1, HA1, ER, CO2, and CD2) that was strongly neutralized.

Determination of IHNV serotypes. A comparison of the n.i. (Tables 4 and 5) indicated that there were sufficient immunological differences among the IHNV isolates to define variants (6, 25). To determine whether these differences also constituted a basis for distinguishing serotypes, we used two methods of analyzing the data. In the first method, antibody neutralization titers were determined and the antigenic difference values, $1/r$, were calculated by the method of Archetti and Horsfall (4). If $1/r$ was greater than 2 (less than 50% antigenic similarity), then the compared viruses were serologically distinct; if $1/r = 1$, then the compared viruses were serologically identical. The two-way cross-neutralization antibody titer ratios were determined for IHNV RB1 and IHNV CO2 with antisera to IHNV RB1 and CO2 and to their respective G proteins (Table 6). At a 10-fold n.i. (n.i. = 1) for antisera to IHNV RB1 and IHNV CO2, the $1/r$ value (antigenic difference) was 0.79. The $1/r$ value was 1.37 for antisera to IHNV RB1 G protein and IHNV CO2 G protein at a 50-fold n.i. (n.i. = 1.7). When the two isolates were compared by using antiserum to IHNV RB1 G protein and antiserum to IHNV CO2 G protein, the $1/r$ value was 1.00. Thus, no serotypic difference between IHNV CO2 and RB1 was seen with polyclonal antiserum to either the whole viruses or their respective G proteins.

The second method used to determine whether there were different serotypes of IHNV was based on the work by Kapikian et al. (16). In this case, serotypes were defined by the absence of neutralizing activity on a heterologous virus with a 20-fold excess of an antiserum concentration that would normally neutralize 10-fold more homologous virus. IHNV RB1 was neutralized significantly (n.i. = 2.4) at a dilution of 1:100 of anti-IHNV RB1 serum. At a twofold excess dilution of the limit antiserum to IHNV RB1 (1:50 dilution), the weakly neutralized IHNV isolates (LE, DW2, and NS) were all significantly neutralized (Table 4). With antiserum to IHNV RB1 G protein (Table 5), the limit neutralizing dilution for IHNV RB1 was 1:40. At a 1:20

dilution, again only a twofold excess of the limit dilution of antiserum, even the previously unneutralized IHNV NS and poorly neutralized DW3 isolates were significantly neutralized.

At a 1:20 dilution of anti-IHNV CO2 serum, there was significant neutralization of IHNV CO2. Two of three poorly neutralized isolates (LE and DW3) were all significantly neutralized at a 1:5 dilution of this antiserum (data not shown), which was a fourfold increase in antiserum (Table 4). Although not shown in the table, at a 1:4 dilution (fivefold increase) in antiserum, DW2 was significantly neutralized (n.i. = 2.0). IHNV CO2 was significantly neutralized (n.i. = 1.7) with antiserum to IHNV CO2 G protein at a minimum dilution of 1:200 (Table 5). The three isolates (LE, DW2, and DW3) that were poorly neutralized by this antiserum at a 1:200 dilution were significantly neutralized (LE, n.i. = 1.8; DW2, n.i. = 2.0; and DW3, n.i. = 3.0) at a 1:10 dilution, which is 20 times more antiserum (data not shown). Again, a comparison of the neutralization of the most divergent (least neutralized) groups of IHNV with four different antisera showed less than serotypic differences.

DISCUSSION

Ten isolates of IHNV were compared serologically; four of those were used to challenge fish immunized with the G protein from the reference vaccine strain from Round Butte Hatchery (Table 1). The challenges were performed at four virus concentrations from approximately 10^2 to 10^5 $TCID_{50}$/ml. The fish were vaccinated both by immersion and by intraperitoneal injection of purified G protein.

The IHNV envelope G protein has previously been shown to induce protective immunity in fish against challenge with homologous (8) or low concentrations of heterologous (9) types of virulent IHNV. Also, the IHNV envelope G protein was solely responsible for inducing neutralizing antibody and protective immunity in the host, as has been shown previously with the mammalian rabies and vesicular stomatitis rhabdoviruses (7, 17, 26).

Immersion or inoculation vaccination with IHNV G protein induced protective immunity in very small fish (0.4 g). There was no apparent difference in protection obtained by the different routes of antigen exposure, a result that indicated that immersion was an effective and practical method of vaccination. The only case in which immersion vaccination was not as effective as direct G protein injection was in the cross-challenge experiment with IHNV HA1, which is the most virulent strain tested (Table 3). At very high virus concentrations, good levels (RPS > 60) of protection were achieved in challenges with IHNV ER. Moderate protection (RPS = 50 to 60) was achieved at high concentrations of IHNV CO2 and CD2. At the lower virus concentrations which mimic the virus levels encountered in the environment and hatchery (22), good protection was seen with IHNV ER, CO2, and CD2; moderate protection was observed with HA1. Moderate to low levels of protection (RPS = 10 to 60) were observed in fish challenged with high concentrations of IHNV HA1. These findings extend earlier work that demonstrated significant protection in immunized fish to low challenge concentrations of heterologous viruses (9).

Vaccine-induced protection was clearly demonstrated when the challenge virus dose was sufficient to produce an LD_{50} but not so great as to overwhelm the immune system. At IHNV concentrations of 100 and 1,000 $TCID_{50}$/ml, the immunized fish were completely protected. At virus concentrations of 10,000 to 100,000 $TCID_{50}$/ml, there was still significant protection in the vaccinated fish, yet control unimmunized fish were killed at levels of 50 to 100% (Table 3). Thus, statistically significant protection was afforded by the G protein vaccination.

A serological comparison of 10 distinct IHNV isolates representing all five biochemically defined types from different geographic locations was made in this report. The n.i. attained by four different antisera did not distinguish any serotypic differences (Tables 4 to 6). However, these antisera did define two groups: a group of readily neutralized IHNV isolates including RB1, CO2, ER, CD2, HA1, and TA1, and a group of less readily neutralized virus isolates including DW2, DW3, LE, and NS. Among the less readily neutralized virus group, all were type 3 IHNV viruses with the exception of LE, a type 2. There was one type 3 isolate, ER, which was readily neutralized by all antisera. This isolate was also neutralized by monoclonal antibodies to RB1 and to SRCV, an IHNV isolate obtained from chinook salmon in California (3a). The LE isolate was least neutralized by antisera to IHNV CO2 and its G protein. The other three IHNV isolates, DW2, DW3, and NS, were neutralized to an equivalent extent by all the antisera. No clear correspondence of biochemical type to n.i. was evident. Therefore, differences in the N protein, which determines biochemical type (Table 2), were not related to n.i. These results indicate that the less readily neutralized virus isolates are variants of IHNV which have minor serological differences (25).

The IHNV RB1 and CO2 isolates were suspected of being the most distinct serologically from an early report by McCain et al. (21) and because of the difference in apparent molecular weight of the G protein (12). A comparison of the cross-neutralization data between these two isolates indicated that IHNV CO2 and RB1 were highly related and thus constituted a single serotype (Table 6). Using the one-way cross-neutralization method based on the method of Kapikian et al. (16), all 10 IHNV isolates were compared. By this method, all the isolates, including the moderately neutralized group of IHNV isolates, belong to a single serotype. Thus, although there were variations in n.i. with the four antisera, only one serotype was defined for IHNV.

These experiments clearly indicated that the viral G protein alone was able to confer immunity and extended previous results with heterologous viral challenges to show that immersion vaccination provides protection comparable to that of inoculation vaccination. Another point established by these experiments was that the IHNV variants (type 1 to type 5) have a conserved antigenic site(s) and that a vaccine based on a single type of IHNV was possible. Such a prototype vaccine has been described previously (11). Fish in any geographic area may be protected from endemic as well as exotic IHNV types, and the development of a polyvalent vaccine may not be necessary.

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MANUSCRIPT # 15

Bacterially Expressed Nucleoprotein of Infectious Hematopoietic Necrosis Virus Augments Protective Immunity Induced by the Glycoprotein Vaccine in Fish

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Bacterially Expressed Nucleoprotein of Infectious Hematopoietic Necrosis Virus Augments Protective Immunity Induced by the Glycoprotein Vaccine in Fish†

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The ribonucleoprotein gene of infectious hematopoietic necrosis virus (IHNV) has been expressed in *Escherichia coli* as a *trpE* fusion protein. This viral protein does not induce protective immunity to lethal IHNV infection in fish, and virus-neutralizing antibodies do not react with this viral protein. However, when it was administered with a bacterial lysate containing a region of the IHNV glycoprotein, there was enhanced resistance in immunized fish to lethal virus infection.

Infectious hematopoietic necrosis virus (IHNV) belongs to the family *Rhabdoviridae* and is a major viral pathogen of young salmon and trout. Mortality rates among hatchery-reared fish can be as high as 90%. Thus, viral epizootics can place severe economic restraints on aquaculture production, and a safe, effective vaccine was needed by the industry. A subunit vaccine composed of the glycoprotein of IHNV has been developed by recombinant DNA techniques (7). Its effectiveness with many different isolates of IHNV has been demonstrated (5). However, there were virulent IHNV isolates which were resistant to the initial protection provided by the vaccine, and laboratory studies indicated that there was no protection against high virus concentrations for some virus isolates (unpublished data). Thus, the vaccine needed further examination. This report describes the effect of the IHNV ribonucleoprotein in enhancing the protection induced by the subunit vaccine.

The genome of the virus consists of an unsegmented, single-stranded RNA of about 11,000 bases encoding five virion proteins: N (nucleoprotein), M1 and M2 (matrix proteins), G (glycoprotein), and L (polymerase) (12). In addition, IHNV transcribes a sixth mRNA for a non-virion-associated protein, NV, of unknown function, which is a feature not described for other rhabdoviruses (13).

Recent studies in our laboratory have shown that the glycoprotein is the only viral protein capable of eliciting a neutralizing-antibody response against the virus in vitro (6). Further experiments have shown that purified virion glycoprotein was capable of producing a protective response to a lethal virus challenge in salmonid fish (5). A region of the glycoprotein gene from the Round Butte (RB) strain of IHNV was expressed in *Escherichia coli*, and this expressed protein was effective in inducing protective immunity in fish (7). The two recombinant plasmids which were found to produce a region of the IHNV glycoprotein gene in bacteria as *trpE* fusion proteins were p52G and p618G. Both plasmids contained a fragment of the glycoprotein gene encoding amino acids 336 to 444 (329 bp), which had been ligated in frame to the *trpE* gene of a pATH expression vector (11). They differed by additional glycoprotein gene sequences which were ligated out of frame to the 3′ ends of the 329-bp fragments. The molecular masses of the fusion proteins expressed by p52G and p618G were 49 and 48 kDa, respectively. Both of these plasmids produced bacterial lysates which were effective as vaccines. However, laboratory tests of the vaccine with some heterologous strains of IHNV indicated that the immune protection was observed only when the immunized fish were challenged with low concentrations of virus. At high virus concentrations, the fish showed no evidence of protection. The observed differences in virulence among virus strains were not due to major variations in the antigenic structure of the viral glycoprotein, because studies with polyclonal neutralizing rabbit antisera to IHNV had indicated that there was only one serotype of IHNV (6, 15). Monoclonal antibodies did show that there were conserved as well as differing neutralizing epitopes on the viral glycoprotein (17).

Several reports have indicated that in other pathogenic virus systems, internal viral antigens can contribute significantly to the induction of protective immunity (4, 18). The IHNV nucleoprotein had been shown to be antigenically similar among the viral strains (10). Thus, the role of the IHNV nucleoprotein in the induction of a protective immune response was examined. We demonstrate here that immunization with the IHNV nucleoprotein and the IHNV glycoprotein can lead to increased protection against lethal IHNV infection.

Construction of pLON-3. A full-length N gene was constructed by ligating two clones, pN2216 and pN144, at the *Stu*I site. The pN2216 clone had been mapped to the 3′ end of the N gene, and the pN144 clone had been mapped to the 5′ end of the N gene (8). The completed N gene was ligated into the *Pst*I site of the pGEM vector as shown in Fig. 1, and the construction was verified by DNA sequence analysis of the plasmid pGEM/N. Examination of the N gene sequence indicated that the first 107 bp of the gene were noncoding. In order to generate an expression plasmid for the N gene in *E. coli*, this noncoding region was deleted. A 296-bp fragment from *Xho*I to *Pvu*I and a 918-bp fragment from *Pvu*I to *Hin*dIII were ligated into the *Sal*I-*Hin*dIII-digested pATH-3 expression vector. The construction was designed to insert the N-coding region in frame with the *trpE* structural gene. Verification of this in-frame ligation to the *trpE* gene was made by direct plasmid DNA sequence analysis of pLON-3.

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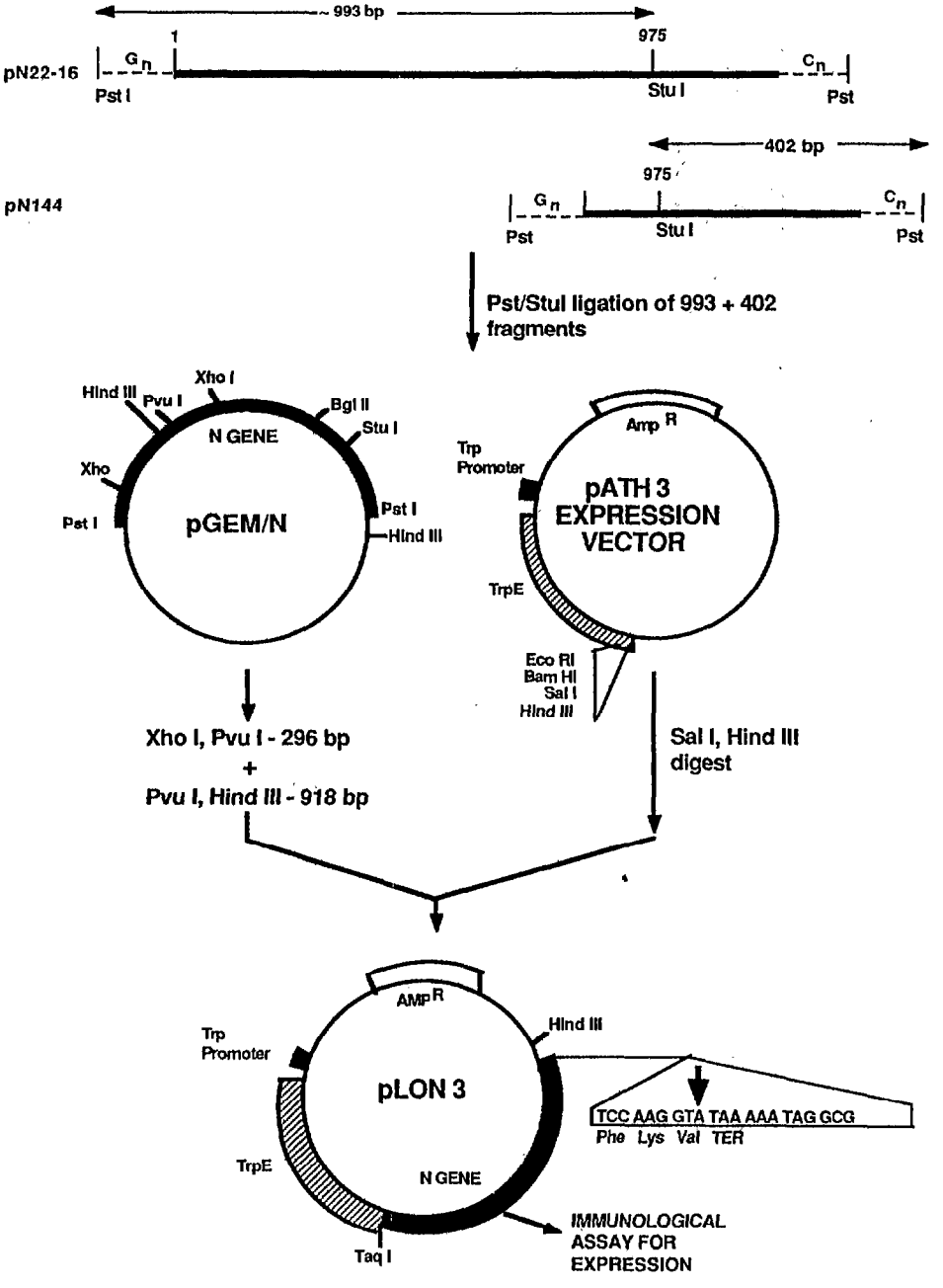

FIG. 1. Construction of the plasmid pLON-3 for expression of the IHNV nucleoprotein in *E. coli*. To construct pGEM/N, a 993-bp fragment from the clone pN22-16 from the left *Pst*I site to the *Stu*I site was purified and ligated to a 402-bp fragment taken from the *Stu*I site to the right *Pst*I site in pN144. These fragments were ligated to *Pst*I-digested pGEM to yield pGEM/N. The large arrow indicates the *Stu*I site lost in pLON-3.

An examination of the 3′ end of the insert indicated that a region of the N gene from the *Stu*I site to the end of the gene had been deleted and vector sequences had been inserted to yield a truncated nucleoprotein. The sequence and the encoded amino acids in this region are shown in Fig. 1; the N gene sequence ends immediately at the *Stu*I site, which is shown by the arrow. Appended to this are nucleotide sequences from the expression vector, which provided a termination codon immediately after the amino acid valine. No additional amino acid was added to the N gene at the *Stu*I site. Transformed bacterial colonies were tested for expression of the N protein by positive staining with anti-IHNV sera.

Expression of IHNV nucleoprotein in *E. coli*. The bacteria hosting the pLON-3 plasmid were grown in broth culture and induced with indoleacrylic acid. Analysis of the bacterial lysate indicated that the viral N gene was expressed as a *trpE*-N fusion protein of approximately 65,000 Da, which was composed of the *trpE* gene (37,000 Da) and the 270 amino acids of the N gene present in pLON-3 (about 29,000

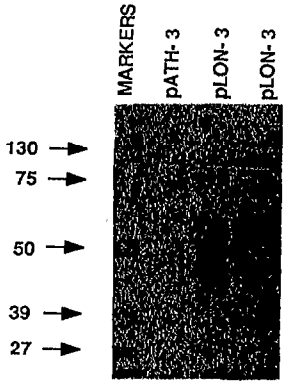

FIG. 2. Western blot of N expression in *E. coli*. Bacterial lysates were prepared for cultures hosting the plasmid pATH3 or pLON-3. The cells were grown in the presence of ampicillin overnight, at which time indoleacrylic acid was added to the culture for 4 h. The cells were collected, and lysates were prepared as described by Gilmore et al. (7). A Western blot of the bacterial lysates shows that anti-IHNV sera did not detect any distinct protein in cells hosting the pATH3 expression vector with no insert. In lysates from two different cultures of bacteria hosting pLON-3, a distinct band at 65,000 Da was detected. In some culture lysates, a smaller band at 48,000 Da was also observed. It is possible that this band represents a breakdown product of the 65,000-Da *trpE*-N fusion protein. Numbers on the left are molecular masses (kilodaltons).

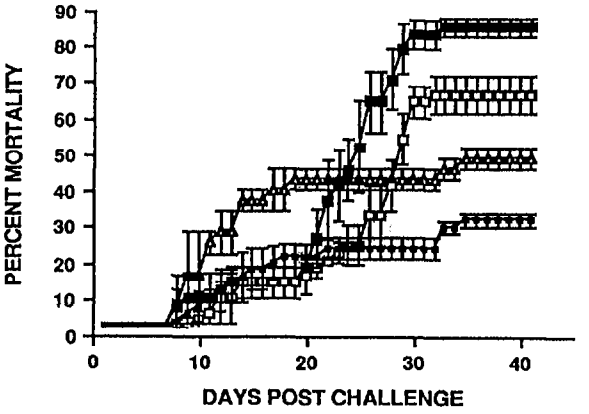

FIG. 3. Mortality rate of fish challenged with the IHNV RB1 strain. Rainbow trout fry (0.4 g) were immunized with a bacterial lysate containing the plasmid p52G or pLON-3 (N) or an equal mixture of lysate from both preparations. All immunized fish were immersed in a bacterial lysate brought to a concentration of 3 mg/ml, 100 fish per 25 ml of solution. The control fish were not immunized. Thirty days after the vaccination, the fish were challenged with approximately 1,000 to 10,000 50% tissue culture infective doses of IHNV. The tests were in three replicates of 30 fish each. Each point has been plotted as an average with the range also shown. The cumulative percent mortality for each group is plotted against the number of days after viral challenge. Symbols: ■, C; □, N; △, G; ◆, G + N.

Da) (Fig. 2). In some of the bacterial fermentations, an additional band at 48,000 Da was detected as a breakdown product of the *trpE*-N fusion protein.

Induction of protective immunity in nucleoprotein-primed fish. To provide more extensive immune protection for fish against different isolates of IHNV, the role of the viral nucleoprotein in immune protection was examined. Because it was difficult to prepare enough purified viral nucleoprotein from purified virions by standard methods, the N gene was expressed in bacteria and the crude bacterial lysate was used to immunize fish. Under viral challenge conditions that resulted in the death of 83% of the control nonimmunized fish, 47.2% of the fish immunized with the p52G vaccine died (Fig. 3). The fish that received only the pLON-3 bacterial lysate were immune (64.5%) to virus challenge at a level similar to that obtained with a bacterial lysate containing no viral protein (7). However, when fish were immunized with a bacterial lysate mixture containing equal quantities of the *trpE*-G fusion protein from p52G and the *trpE*-N fusion protein from pLON-3, a marked increased in protection was observed. This group exhibited a lower mortality rate, and the final mortality at the end of 40 days was 29.9%, an increase in protection of 17.3%. The tests were carried out with three replicates of each group, and the graph shows the averages and the ranges of the results (Fig. 3).

The amount of vaccine used in each immunization group was 25 ml (3 mg of total protein per ml) per 100 fish. Each fish weighed approximately 0.4 g at the time of immunization. The fish receiving only the *trpE*-G fusion protein or only the *trpE*-N fusion protein were immunized in a bacterial lysate which had been adjusted to 3 mg of total protein per ml. The fish receiving a mixture of the two different fusion proteins were immersed in a solution containing 1.5 mg of total protein per ml from each lysate so that the total protein concentration was 3 mg/ml. The contribution of expressed viral protein in each mixture was approximately 10% of the total protein.

Similar results were obtained when a heterologous IHNV isolate was used to challenge the fish (Table 1). Additional studies showed that the enhanced protection observed with the combined G and N subunit vaccines was also found for two other isolates of IHNV. The genes encoding the IHNV N and G proteins were cloned from the RB type 1 (RB1) strain of the virus (7, 13). The challenge virus used in these experiments included the 039-82-OSU isolate and the Dworshak 1988 isolate. The N proteins of these isolates can be

TABLE 1. Protection of rainbow trout fry from virulent IHNV by immunization with N and G subunit vaccines

Challenge IHNV strain[a]	Group[b]	No. of fry	Avg specific loss	% Mortality
RB1 (2.2×10^3)	C	17[c]	25.0	83.3
	p52G	17[c]	14.2	47.2
	N	17[c]	19.4	64.5
	G + N	17[c]	9.0	29.9
039-82-OSU (1.17×10^4)	C	45	42.0	93.0
	p52G	45	31.0	68.8
	N	45	41.0	91.1
	G + N	45	21.0	46.6
Dworshak-88 (3.16×10^3)	C	50	17.0	34.0
	p618G	50	20.0	40.0
	N	50	19.0	38.0
	G + N	50	6.0	21.0

[a] Values in parentheses are titers of IHNV used to challenge the immunized fish, given in 50% tissue culture infective doses per milliliter.

[b] C, control (unvaccinated group); N, group receiving pLON-3 lysate; G + N, group receiving both pLON-3 and the indicated G subunit vaccine; p52G and p618G, group receiving G subunit vaccine (clone designation as described by Gilmore [7]).

[c] Three groups of 17 fry each.

distinguished from the N protein of RB1 by differences in electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels, and both are classified as type 2 viruses (9). However, polyclonal anti-IHNV antibody to IHNV RB1 will react with the N proteins of over 100 different IHNV isolates in Western immunoblots (10). This suggests that the enhanced immunoprotection that was observed with the G-N combination vaccine might be observed for a wide variety of IHNV isolates.

Both the p52G and p618G expression clones were used in the studies reported here (Table 1). The p618G plasmid contained a region of the IHNV glycoprotein gene which was identical to that of p52G but encoded additional amino acid residues provided by read-through into the *trpE* gene. This plasmid also induced enhanced protection by the addition of the N subunit vaccine.

In summary, an augmented immune response to IHNV was induced by a bacterial lysate containing the viral nucleoprotein expressed as a *trpE*-N fusion protein. The efficacy of the nucleoprotein was examined by immersion vaccination in fish as small as 0.25 g (Fig. 3) and resulted in 20 to 25% increased protection in the fish immunized with vaccines containing both G and N proteins.

The mechanism(s) by which *trpE*-N-immunized animals were protected is unknown. It has not been possible to examine the immune response of vaccinated fish because of the size of the fish (0.25 to 1.0 g). However, it has been shown that the nucleoprotein of rabies, another rhabdovirus, stimulates the proliferation of rabies-specific T-helper cells, and most T-helper cell lines tested are highly responsive to the nucleoprotein and less so to the G protein (1). Furthermore, internal influenza virion antigens have been shown to generate murine T-helper cells that were able to help the antihemagglutinin antibody responses in vitro (16) and in vivo (18). These findings indicate that the IHNV nucleoprotein might affect the cellular immune response in fish in a similar manner. Another speculation that has been made is that the rabies nucleoprotein activates the cytolytic T cells and that these cells play a major role in the elimination of infectious virus. Likewise, it is possible that cells infected with IHNV were destroyed by IHNV-specific cytolytic T cells primed by the viral nucleoprotein.

When the N protein was analyzed for T-cell antigenic sites with a computer program based on an algorithm for amphipathic helices (3), there emerged 20 peptide segments with amphipathic scores above 8.0. These scores reflect the probability that these peptides act as T-helper lymphocyte binding sites. The peptides with the highest scores were located near the N-terminal and C-terminal thirds of the protein. This result was interesting because we have found that rabbit polyclonal antisera to the IHNV N protein will recognize only N protein regions from the N terminus and C terminus in Western immunoblots (2). Although the computer predictions have been verified only for murine and rabbit T-cell binding sites (14), it does not seem unreasonable to expect that fish lymphocytes will react in a similar manner. The use of synthetic peptides derived from the N protein and identified as T-cell antigenic sites in fish may be useful as adjuvants for the IHNV vaccine.

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SUMMARY

A subunit vaccine to IHNV has been developed by recombinant DNA technology. The vaccine consists of a lysate of *Escherichia coli* which has been transfected with a plasmid, pXL3, encoding an immunogenic region of the viral glycoprotein. Fish immersed in this crude lysate are vaccinated against a lethal dose of the virus in assays covering an LD50 assessment of immunity. Since this vaccine had reached a stage in its development for transfer of this technology to private industry, Oregon State University licensed the technology to MariGenetics, Inc., the only company that offered to take the vaccine to market with diligence. Technology transfer is mandated by the U. S. Department of Energy. MariGenetics will carry out the studies that should lead to licensing of this vaccine by the U. S. Department of Agriculture. It was not possible for the University or Bonneville Power Administration to enter into the practice of licensing and manufacturing the vaccine for commerical development.

In the development of the IHNV vaccine, a number of useful reagents became available to monitor and perhaps control IHNV infections in the Columbia River basin (CRb). The viral genes encoding the nucleoprotein, matrix proteins, and glycoprotein of IHNV were cloned and have been subsequently used to develop *in situ* hybridization probes for the IHNV Life Cycle project. In addition, the expression of different regions of the viral glycoprotein in bacteria has enabled us to determine that fish are restricted in their antibody response to the virus. This finding may provide us with a reason for peristence of this virus among survivors of an IHNV epizootic.

Monoclonal antibodies developed by Sandra Ristow, John Fryer, and Jim Winton will enable us to follow the evolution of the virus in a natural environment. Most importantly, they will provide us with tools to monitor

IHNV in wild populations of fish. Initial studies by the Oregon Department of Fish and Wildlife suggest that the wild populations of fish have a higher rate of endemic infection with IHNV. It may be that IHNV in wild fish is more of a problem than previously thought and these fish may actually be the source for the continued presence of the virus in the system. A much needed study of wild fish should be made by nonlethal sampling. The probes for IHNV which have been developed in this project are a side benefit of the work and provides an excellent example of how research projects can have wide reaching benefits for the fisheries resource.